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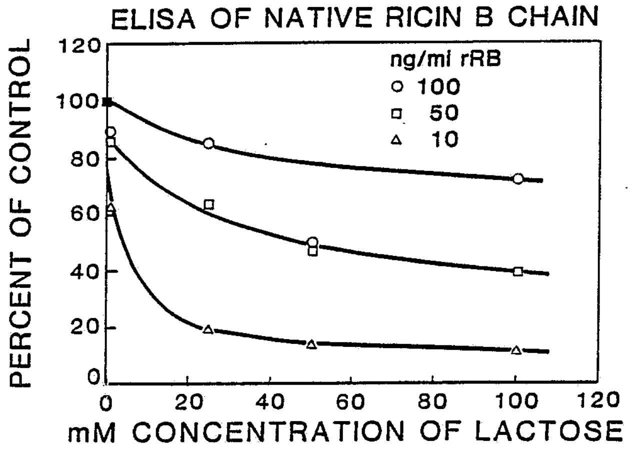
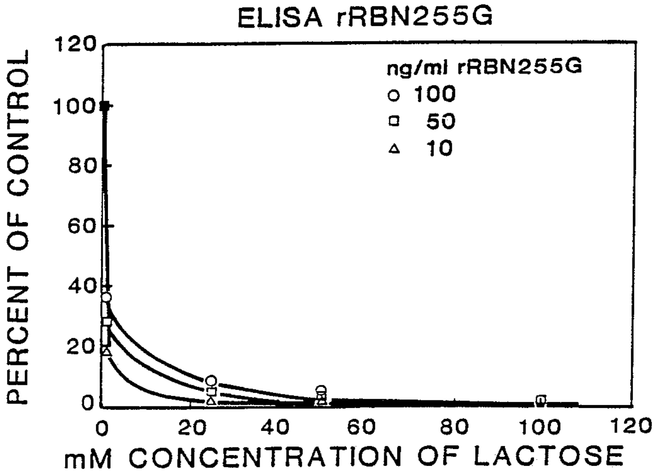

ELISA OF NATIVE RICIN B CHAIN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(57) Abstract

Cloning and expression of ricin toxin, muteins of ricin toxin having reduced galactose binding activity, or subunits thereof is described wherein these molecules are cloned into novel baculovirus transfer vectors and co-infected into insect cells with baculovirus thereby effecting recombination with the transfer vectors producing baculovirus expression vectors capable of infecting insect cells and expressing ricin toxins, muteins, or subunits thereof.

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Production of ricin toxins in a baculovirus-insect cell expression system.

The present invention relates to the field of molecular biology and proteins. In particular, the invention relates to the achievement of expression of ricin toxin proteins using an insect cell/baculovirus expression system.

Numerous different expression systems are now available to the genetic engineer who wishes to express a cloned gene of interest. Generally the goal is the production of high levels of biologically active material. The requirements for the expression system chosen to accomplish this goal are dependent upon the nature of the protein to be expressed.

The value of utilizing prokaryotic host vector systems for the synthesis of desirable eukaryotic proteins is diminished by certain limitations inherent in such systems. For instance, the mRNA transcript or protein product of such systems may be unstable in the prokaryote. In addition, before a protein will be synthesized within a prokaryotic cell, the optimal DNA sequence introduced into the microorganism must be free of intervening DNA sequences, nonsense sequences, and initial or terminal sequences which encode for polypeptide sequences which do not comprise the active eukaryotic protein. Further, some eukaryotic proteins require modification after synthesis (e.g., glycosylation and all membrane associated processing) to become biologically active, and prokaryotic cells are generally incapable of such modifications.

Various nonviral eukaryotic host vector systems are also available for the expression of heterologous proteins. Certain limitations are inherent in each of these systems as well. For example, high levels of expression are frequently difficult to obtain in yeast systems where autonomously replicating vectors may be unstable. Additionally, glycosylation patterns in yeast differ from those in higher eukaryotes.

Limitations encountered with mammalian host vector systems include difficulties in host cell culturing and its scale-up. The expense of mammalian cell culture media as well as a requirement for serum often restricts its use on a large scale and complicates the use of these systems for production of parenterally administered pharmaceuticals. Furthermore, levels of expression in these systems are generally substantially lower than that obtained in prokaryotic or viral expression systems.

The use of viruses in eukaryotic host-vector systems has been the object of much speculation. However, some viral vector systems also suffer from significant disadvantages and limitations which diminish their utility. For example, a number of eukaryotic viral vectors are either tumorgenic or oncogenic in mammalian systems and create potential health and safety problems associated with resultant gene products and accidental infection.

The baculovirus expression vector system involved in the instant invention overcomes many of the above-mentioned limitations. Baculoviruses are insect pathogenic viruses which, until recently, were studied mostly for their potential use as viral insecticides for control of agriculturally important insect pests. Because certain baculoviruses are highly virulent for pest insects, some of the most promising have been commercially developed and are used as biological pesticides (Miltenburger and Krieg 1984 Bioinsecticides:II: Baculoviridae. Adv. Biotechnol. Processes 3:291; Granados, R.R. and Federici, B.A. eds. The Biol. of Baculoviruses Vol II, Boca Raton, FL: CRC Press, Inc. 1986). Baculoviruses are very stable and are able to persist for longer times in the environment than other animal viruses. This unusual biological stability is the result of a unique association of the infectious virus particles and a viral occlusion that is a crystalline assembly of a viral encoded structural protein called polyhedrin. Late in viral replication, baculovirus particles become embedded in a protein occlusion composed of the polyhedrin protein. Insects acquire a baculovirus disease by ingesting the occluded virus (OV) which contaminate their food supply. The polyhedrin matrix protects the virus particles in the environment and during their passage through the foregut of the insect. In the insect midgut, the alkaline pH activates the dissolution of the polyhedrin crystalline matrix resulting in the release of many viruses. The virus become absorbed by the midgut epithelial cells and initiate the infection process.

There is a second infectious form of nuclear polyhedrosis viruses (NPVs), known as the extracellular or nonoccluded virus (NOV) form, which is generated by the budding of viral nucleocapsids through the plasma membrane of the infected cells. NOVs are responsible for spreading a secondary infection via the hemolymph of the insect. It is the NOV form of the virus which is infectious in insect cell cultures; the occluded (OV) form is not infectious in cell cultures since dissolution of the crystalline matrix occurs only at high alkaline pH (i.e., pH 10.5).

The formation of NOVs and OVs occurs in a biphasic manner during the infection process. NOVs are abundantly produced before occlusion is initiated. During a typical synchronous infection of fully permissive cell lines, the majority of NOVs are produced between 12 and 24 hr post-infection (p.i.). The synthesis of polyhedrin is initiated at 20 hr p.i. and does not reach maximal levels until 48 to 72 hr p.i. The significance of this temporal regulation with respect to the expression vector system is that foreign gene products that may have adverse effects on the cell should not diminish the production of progeny NOVs to be used for further infection.

Of the 450-500 species of known baculoviruses, practically all encode a polyhedrin protein. As previously discussed, the viral occlusion is a paracrystalline assembly of a polyhedrin monomer which, for most viruses, has an average molecular weight of 28,000-30,000 (Summers, M.D. and Smith., G.E., 1978 Virology 84:390). Baculoviruses are unique among animal viruses, not only in the protective function of the viral occlusion in the viral life cycle but also because the polyhedrin gene is the most highly expressed eucaryotic virus gene known. The polyhedrin protein can accumulate to greater than 1 mg/ml of infected cultured insect cells (70-75% of the total cellular protein) or can comprise up to 25% of the total protein of an infected insect. Although very highly expressed, neither the polyhedrin gene nor its protein is essential for viral infection or replication in cultured insect cells or insects, thus making the polyhedrin gene an ideal target for genetic manipulation.

The most extensively studied baculovirus is the Autographa californica nuclear polyhedrosis virus (AcNPV). The Autographa californica host for AcNPV is a moth commonly referred to as the alfalfa looper. Studies of the physical and functional organization of the AcNPV genome have resulted in the mapping, cloning, and sequencing of the AcNPV polyhedrin gene and its regulatory sequences (Iddekinge et al. 1983 Virology 131:561; Smith et al. 1983 J. Virol. 46:584). Not only does the polyhedrin gene exhibit a strong promoter, but expression can continue late in infection well beyond the point of repression of nearly all other baculovirus and host genes.

The genetic engineering of the baculovirus polyhedrin gene for high level expression of a heterologous protein, in this case, recombinant human β-interferon was first reported by Smith et al. (Mol. Cell. Biol. 3(12):2156-2165 (1983)). Since then, human interleukin 2 has been expressed in insect cells by a baculovirus expression vector as described by Smith et al. (Proc. Natl. Acad. Sci. USA 82:8404-8408 (1985)). Recently, the synthesis of functional human T-cell leukemia virus Type I p40^{x} protein using a baculovirus expression vector has been reported (Jeang, K.T., et al., J. Virol. 61:708-713 (1987). Other heterologous proteins that have been expressed in this system are summarized in Summers et al. ("Genetic Engineering of the Genome of the Autographa californica nuclear polyhedrosis virus," Banbury Report: Genetically Altered Viruses in the Environment," 22:319-339 Cold Spring Harbor Laboratory (1985)).

The baculovirus expression system has several advantages for the expression of foreign genes in comparison to other prokaryotic, yeast or mammalian cell expression vector systems. First, high levels of expressed proteins are possible. Greater than 1.0 mg per ml of polyhedrin protein is normally produced in infected cells. Another non-essential occlusion-related viral protein, p10, is also abundantly produced and its promoter has reportedly been used to drive foreign gene expression (D.W. Miller et al. in Genetic Engineering Principles & Methods 8:277-298, Setlow and Hollaender, eds. New York: Plenum Press, 1986). Using the polyhedrin gene promoter, heterologous gene expression levels never reach polyhedrin levels but are usually in the range of tens to hundreds of micrograms per ml (Summers et al., 1985, p.321, supra). Secondly, in contrast to those produced in bacterial cells, recombinant proteins produced in insect cells may be co- and post-translationally processed in a manner similar to what occurs in mammalian cells. In at least one case, glycosylation of IFN-β in infected insect cells has been reported (G.E. Smith et al. (1983) supra). Whereas about 40% of the natural IL-2 produced in human Jurkat cells is not glycosylated, there was no evidence of any glycosylation of the recombinant IL-2 produced in insect cells (G.E. Smith et al., (1985) supra). In addition, correct cleavage of mammalian secretory signal peptides has been observed (G.E. Smith et al., (1983) supra ; G.E. Smith et al., (1985) supra; D.W. Miller et al., (1986) supra).

The expression of naturally occurring toxins which exert their toxic effects by inactivating the large subunit of eukaryotic ribosomes presumably requires either temporal regulation of expression or modified processing so as to protect the cell against potential toxicity in order not to adversely effect the host eukaryotic cells. The baculovirus expression vector system has been tested as an alternative system to produce whole ricin toxin, ricin B chain, and products containing ricin A chain.

The potent ricin toxin and the Ricin communis agglutinin (RCA) are two major lectins found in the beans produced by the castor oil plant (Ricinus communis). These proteins have been extensively studied, including determination of the complete amino acid sequence for one form of ricin toxin (for review, see Olsnes, S. and Pihl, A. (1982a) in Molecular Action of Toxins and Viruses, pp. 51-105, Cohen and vanHeyningen (eds.), Elsevier Biomedical Press; Olsnes, S. and Pihl, A. (1982b) Pharmac. Ther. 15, 355-381). Each of these lectins contains two different glycosylated subunits (A and B), each of approximate molecular weight 30,000, linked via a disulfide bridge. The toxin contains one subunit of each type, whereas the agglutinin contains two of each. The A subunit acts to catalytically inactivate eukaryotic ribosomes, whereas the B subunit binds to cell surface galactose-containing structures and facilitates entry of the A subunit into the cytoplasm.

In recent years, both of the toxin subunits have been used extensively as components in hybrid toxins targeted to specific cells (Olsnes, S. et al. (1982b) supra; Moller, G. (ed.) (1982) Immunol. Rev., 62, Vitetta, E.S. et al. (1985) Cell, 41:653). Whereas the A subunit provides the essential catalytic cytotoxicity to these molecules, the B subunit may also be used to enhance membrane transport (Vitetta, E., et al. (1983) Proc. Natl. Acad. Sci. (USA) 80, 6332).

Ricin subunits have been individually expressed in bacterial hosts. In general, the ribosomes of prokaryotic cells are resistant to enzymatic inactivation by ricin toxin A and intact ricin comprising ricin toxin A and B chains. However, it is important to note that ricin fragments are thought to be toxic in E. coli. European Patent Publication No. 237,676, published September 27, 1987, discloses the expression of the ricin A subunit in E. coli. European Patent Application No. 86301227.4, filed February 20, 1986 and PCTWO/88/00593, filed February 24, 1988 describe the expression of ricin B subunit or its muteins in E. coli. M. O'Hare et al. (Febs Letts. (1987) 216:73) more recently reported the expression of ricin A subunit in E. coli. There are no known disclosures of full length ricin toxin expression in prokaryotic hosts.

There are no reports known to the inventors at the time of this filing of the expression of full length toxin in a eukaryotic system. This is not surprising since upon internalization, ricin A functions to catalytically inactivate the large subunits of eukaryotic ribosomes thereby causing cell death (Olsnes, S. et al. (1982a) supra; Olsnes, S. et al. (1982b), supra). Vitetta et al. (Proc. Natl. Acad.

Sci. (in press), 1987) have described the expression of ricin B chain as a secreted product from monkey COS-M6 cells, although the amount of ricin B produced is extremely low.

The invention relates, in one respect, to methods for producing by recombinant DNA technology biologically active ricin toxin, ricin toxin subunits or isotoxins thereof and proteins having an amino acid sequence substantially equivalent to these molecules, including Ricin communis agglutinin, and expressing these molecules using recombinant baculovirus expression vectors in suitable host insect cells. Accordingly, one aspect of the invention relates to growing the infected insect cells under suitable conditions to produce the desired recombinant ricin toxin, subunits thereof or related molecules and recovering the biologically active polypeptide, dimerized polypeptide or subunits thereof from the culture medium.

A second aspect of the invention is directed to recombinant baculovirus transfer and expression vectors for producing active muteins of ricin isotoxins D, E and E1, or their subunits, and Ricin communis agglutinin, having reduced galactose binding activity.

In another aspect, the invention is directed to recombinant baculovirus expression vectors which are capable of affecting the expression of ricin toxin, ricin toxin subunits or proteins having an amino acid sequence substantially equivalent to that of ricin toxin or to the host insect cells infected with such vectors, and to cultures thereof.

One aspect of the invention concerns recombinant baculovirus expression vectors in which ricin toxin, ricin toxin subunits and related molecules are expressed under the transcriptional control of a baculovirus promoter. In one aspect of the invention the baculovirus promoter is the polyhedrin gene promoter.

Another aspect of the invention concerns recombinant baculovirus expression vectors in which the DNA encoding ricin toxin, ricin toxin subunits or proteins having an amino acid sequence substantially equivalent to that of ricin toxin is in proper translational reading frame with the DNA encoding a heterologous secretory signal peptide.

Also, aspects of the invention are the recombinant baculovirus transfer vectors which are used to transfer the desired recombinant gene into the baculovirus genome.

Figure 1 is a ribbon representation of the ricin backbone. The A chain is in the upper right and the B chain at lower left. The two lactose moieties bound to the B chain are each represented as pairs of discs. The chains have been separated slightly to facilitate viewing. The disulfide bond linking the chains is indicated in the lower right portion of the molecule.

Figure 2A shows the position and sequence of oligodeoxyribonucleotide primers used to create SalI and PvuII sites in the amino terminal region of the ricin B sequence.

Figure 2B shows the position and sequence of oligodeoxyribonucleotide primers used to create an XbaI and SacII site in the carboxyl terminal region of the ricin B sequence. The numbers at the right of the figures are nucleotide numbers in the complete ricin sequence. Amino acids are designated by the single letter observations approved by the IUPAC-IUB Commission on Biochemical Nomenclature.

Figure 3 shows the DNA sequence of ricin toxin D.

Figure 4 shows the DNA sequence comparison between the recombinant baculovirus transfer vectors pAcC1-C5. The carrots represent restriction endonuclease cleavage sites.

Figure 5 shows the vector constructs used to produce ricin B muteins.

Figure 6 shows the vector constructs used to produce the ricin B double construct·46/255.

Figure 7 shows cytotoxicity assays of supernatants of baculovirus expression system products.

Figure 8 shows a Western blot analysis of insect cell expression products after infection with a recombinant baculovirus containing full length ricin toxin gene sequences.

Figure 9 shows a Western blot analysis of insect cells expression products after infection with recombinant baculovirus containing ricin B gene sequences.

Figure 10 shows the binding of native ricin B to asialofetuin coated on the wells of 96-well dishes in the presence or absence of lactose.

Figure 11 shows the binding of ricin B mutein 255 at different concentrations to asialofetuin in the presence or absence of lactose.

Modes for Carrying Out the Invention

A. Definitions for Ricin Toxin

Ricin toxin is best defined by describing what is known in the scientific literature. Ricin toxin (RT or ricin) is a naturally occurring toxin composed of an enzymatically active, cytotoxic "A" amino acid sequence or subunit, and a "B" sequence or subunit, which is presumed to be responsible both for attaching the "A" subunit to a target cell to be killed, and to aid in the translocation or transport of A subunit into the cytoplasm (see Olsnes, S. et al. (1982a&b) supra). Other examples of such toxins include diphtheria toxin and the exotoxin from Pseudomonas aeruginosa. Other toxic proteins, such as, for example, those derived from Phytolacca americana (PAPI, PAPII, and PAP-S) and gelonin show in vitro activities comparable to the "A" subunits of the above toxins, but are much less active in vivo, presumably due to the absence of a "B" subunit.

The "ricin" peptides of the present invention are derived from the seeds of Ricinus communis, commonly known as castor beans. Two similar proteins (often called lectins) are extractable from these seeds: the above-mentioned ricin and Ricin communis agglutinin (RCA). Both proteins contain A and B portions; however, the A and B portions do not comprise a single peptide. The A portions or these moieties are capable of catalytically inactivating the large subunit of ribosomes in vitro and the mechanism of ricin for in vivo cytotoxicity is believed to reside in this capacity for ribosome inactivation. Ricin and RCA appear to be highly homologous (Cawley, D. B., et al, Arch. Biochem. Biophys. (1978) 190:744) but differences exist. RCA is dramatically less toxic, and appears to exhibit some characteristics corresponding to those expected of a dimer of ricin.

Careful fractionation of castor bean extracts shows the presence of several ricin isotoxins. The distinction between ricins D and E has been previously disclosed (Mise, et al., Agric Biol Chem (1977) 41:2041-2046; Wei, et al., J Biol Chem (1978) 253:2061-2066; Lin, et al., Eur J Biochem (1980) 105:453-459; Genaud, et al., J Immunol Meth (1982) 49:323-332). Ricin D has a pI near 7.4 and a high affinity for agarose; ricin E has a pI near 8.8 and a low affinity for agarose. There are several reports of purported isotoxins which have been shown to be more acidic forms of ricin D (Olsnes, et al., J Biol Chem (1974) 249:803-810; Ishiguro, et al., Toxicon (1976) 14:157-165; Cawley et al., Arch Biochem Biophys (1978) 190:744-755).

The differences in properties between ricins D and E seem to reside in the B chain (Funatsu et al., Agric Biol Chem (1978) 42:851-859, Araki, T. et al. (1987) Biochem Biophys. Acta, 911:191). The RTA chains from ricins D and E are identical in composition, pI, and apparent molecular weight. In the ricin toxins there are two distinct RTA species, RTA1 and RTA2. These isoenzymes differ in molecular weight by SDS-PAGE and in carbohydrate content, and can be resolved by ion exchange chromatography with a very shallow salt gradient (Olsnes, et al., Biochemistry (1973) 12:3121-3126, Foxwell, B.M.J., et al. (1985) Bioch. Biophys. Acta, 840:193).

The ribotoxin most similar to a ricin E isotoxin preparation was designated as ricin E1 and the novel ribotoxin was designated ricin E2. Ricin E2 has a pI identical to that of ricin E1. Compared to ricin E1, it is 1% as toxic to mice and 2-4% as toxic to cultured cell lines, is bound to agarose more tightly at moderate to high ionic strength, and is approximately 2 kD larger by SDS-PAGE.

The components of ricin and of RCA have been well characterized on the basis of the extracted materials, and their properties extensively reviewed: Olsnes, S., Perspectives in

Toxicology, A.W. Bernheimer, Ed (1977) J. Wiley & sons, NY, pp. 122-147; Olsnes, S., et al., Molecular Action of Toxins and Viruses, Cohen, et al., Ed (1982) Elsevier, Amsterdam, pp. 51-105. Ricin has an apparent molecular weight of 58,000 daltons and consists of the A chain with a molecular weight of 32,000 daltons and a B chain of molecular weight of 34,700 daltons. RCA is a tetramer which has two A subunits of molecular weight 32,000, and two B subunits of molecular weight 36,000 each. In their native environments, the A and B chains are generally glycosylated. The A and B subunits of both ricin and RCA are linked only by a single disulfide bond, and not by peptide linkage unlike, for example, diphtheria toxin which is found as a single chain peptide. It is also known that both ricin and RCA, though having separate peptides for A and B portions, are each derived from a single chain precursor in each case (Butterworth, H.E., et al., Eur J Biochem (1983) 137:57). This precursor was shown to contain a sequence of 12 amino acids between the A chain (amino terminal) and B chain (carboxy terminal) sequence; PCTUS/88/00197 filed February 7, 1985. European Patent Appplication No. 86308877.9 filed November 13, 1988 shows the ricin A sequence to contain 265 amino acids preceded by a 35 amino acid leader (signal) peptide. It is assumed that upon excision of the dodecameric intervening peptide, the A and B chains remain linked through the single disulfide bond. The cDNA insert in pRT17 corresponds to the composite between the ricin toxin B chain encoded in the DNA disclosed in PCT/US88/00197 (supra) and the ricin A encoding sequences disclosed in European Patent Publication No. 237,676 (supra). This is the DNA, then, encoding the precursor for ricin D. In addition, the cDNA sequence for the ricin toxin precursor and RCA has recently been reported (Lamb, F.I., Roberts, L.M., and Lord, J.M. (1985) Eur J Biochem 148, 265-270; European Patent Application Publication No. 0145,111 to Lord, J.M. et al., June, 1985).

As is the case for all proteins, the precise chemical structure of ricin toxin, its muteins or subunits, depends on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular protein may be obtained as an acidic or basic salt, or in neutral form. All such preparations which retain their activity when placed in suitable environmental conditions are included in the definition. Further, the primary amino acid sequence may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like, more commonly by conjugation with saccharides. The primary amino acid structure may also aggregate to form complexes. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modification may be introduced in vitro. In any event, such modifications are included in the definition so long as the activity of the protein, as defined above, is not destroyed. It is expected, of course, that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the protein in the various assays. Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, and the protein may be cleaved to obtain fragments which retain activity. Such alterations which do not destroy activity do not remove the protein sequence from the definition.

Modifications to the primary structure of ricin toxin, its muteins or subunits, by deletion, addition, or alteration of the amino acids incorporated into the sequence during translation can be made without destroying the activity of the protein. Native ricin and ricin A exist in a number of homologous but not exactly identical forms depending on the plant variety used as source, and even proteins derived from a single plant may exhibit more than one primary structure. Such substitutions, alterations or variations result in proteins having an amino acid sequence which falls within the definition of proteins "having an amino acid sequence substantially equivalent to that of ricin toxin."

In summary, "ricin" refers to proteins having cytotoxic activity which contain both A and B chains, as set forth herein. Conventionally, as described above, ricin is distinguished from RCA in the art. Both ricin D and ricin E contain A and B chains; it appears that the differences in these proteins lies in the B portions.

Muteins

"Ricin B muteins" according to the invention are defined to be substantially similar forms of ricin B or isotoxins thereof according to the invention in that they fulfill the functional definition of facilitating the intracellularization of an associated toxin molecule. The alterations of the galactoside binding sites of the ricin B muteins decrease the affinity of the ricin B muteins according to the invention for galactosides, yet retain, at least partial functional ability to facilitate the intracellularization of an associated toxin molecule. The precise mechanism whereby ricin B or ricin B muteins facilitates translocation of ricin A is unknown.

Operably Linked

"Operably linked" refers to juxtaposition such that the normal function of the components can be performed. Thus, a coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequence can be expressed under the control of these sequences.

Control Sequences

"Control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. Eukaryotic cells including the insect cells of the instant invention appear to utilize promoters and polyadenylation signals.

Expression System

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed with these sequences are capable of producing the encoded proteins. These DNA sequences may also direct the synthesis of the encoded proteins in an *in vitro* cellular environment. In order to effect transformation, the expression system may be included on a transfer vector; however, the relevant DNA may then also be integrated into the viral chromosome to result in a recombinant viral genome.

Cell, Cell Line, Cell Culture

As used herein "cell", "cell line", and "cell culture" are used interchangeably and all such designations include progeny. Thus "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny which have the same functionality as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

Infection

"Infection" as used herein refers to the invasion of cells by pathogenic viral agents where conditions are favorable for their replication and growth. "Transfection" refers to a technique for infecting cells with purified nucleic acids by adding calcium chloride to solutions of DNA containing phosphate or other appropriate agents such as dextran sulfate thereby causing the DNA to precipitate and be taken up into the cells.

Recombinant Transfer Vector

"Recombinant transfer vector" refers to a plasmid containing a "heterologous" gene under the control of a functional promoter (e.g., polyhedrin or p10 promoter) and flanked by viral sequences. The "recombinant expression vector" is formed after cotransfection of the recombinant transfer vector and wild-type baculovirus DNA into host insect cells whereupon homologous recombination occurs between the viral sequences flanking the heterologous gene and the homologous sequences in the wild-type viral DNA. This results in the replacement of wild-type sequences in the virus with the transfer vector sequences between the crossover points. The recombinant expression vector is the recombinant viral DNA containing the desired heterologous gene.

Biologically Active

"Biologically active" refers to retaining the enzymatic or other biological behavior which typifies the function of the protein in its native state. The biological activity of ricin A refers in one aspect to enzymatic activity, i.e., its ability to inhibit protein synthesis in a rabbit reticulocyte *in vitro* translation system (a commercially available system obtainable, e.g., from Bethesda Research Laboratories, Rockville, MD). In addition to being enzymatically active, soluble preparations of ricin A toxin are also capable of exhibiting specific cytotoxic activity when associated with specific binding portions, for example, immunoglobulins, to form immunotoxins or with the ricin B subunit to reconstitute ricin toxin activity. The biological activity of ricin B refers to its ability to facilitate the intracellularization of an associated toxin molecule via cell surface binding to galactose-containing receptors.

Secretory Signal Peptide

"Secretory signal peptide" refers to a sequence of amino acids that functions to transport a protein expressed in insect cells, such as ricin toxin or subunits thereof, outside the cell. A "heterologous secretory signal peptide" is an amino acid sequence not naturally found in association with the protein to be secreted.

B. General Description

B.1.a. Cloning of the Ricin A Coding Sequence

The approach followed to obtain recombinant ricin A has been described in European Publication No. 215,658 published May 25, 1987 and European Publication No. 237,676 published September 23, 1987. It is, briefly, as described below.

A cDNA library was constructed by isolating mRNA from maturing castor bean seeds, and preparing the corresponding cDNA by, in general, conventional methods. The oligonucleotide 5'-GACCATTTCGAC CTACG-3' which complements the mRNA encoding the N-terminal region of the B chain (which is thus just downstream from the A chain codons) was used as primer in synthesizing the single stranded copy; and an oligo dC homopolymeric tail was added to the 3' end to permit oligo dG to be used as primer in double stranding. The resulting double stranded cDNA fragments were then inserted into the PstI site of the cloning vector, pBR322, by annealing homopolymeric oligo dC tails provided by standard tailing methods to the cDNA with the oligo dG tails which are also thus provided on the cleaved vector. The ligation mixture was transformed into *E. coli*. About 5000 successful transformants were screened for hybridization with probe.

The olignonucleotide mixture 5'-GCATCTTCTTGGTTGTCNGGATGAAA GAAATAGGC-3' (wherein N is A, T, G, or C) was used as a probe. This sequence was initially predicted based on the amino acid sequence described in the review by Olsnes, S., et al., *supra*, and verified.

Positive colonies were analyzed by restriction and showed two pattern types--one predicted to be found from ricin A, and the other presumed to be associated with agglutinin A, since it was significantly different from that obtained from ricin A. A colony was obtained which contained the entire sequence for ricin A, as confirmed by sequencing and comparison of the deduced amino acid sequence to that of native ricin A. Plasmid DNA isolated from this colony was designated pRA123, and given number CMCC 2108 in the assignee's culture collection. pRA123 was deposited with the ATCC on 14 August 1984, and has accession No. 39799.

It should be noted that the procedures of the foregoing paragraphs need not now be repeated in order to obtain the desired ricin A encoding sequences. Using methods known in the art, the appropriate sequence spanning approximately 750 nucleotides may be synthesized. (See, for example, Edge, M.D., et al., *Nature* (1981) *292*:256; Nambiar, K.P., et al., *Science* (1984) *223*:1299; or Jay, Ernest, et al., *J. Biol. Chem* (1984) *259*:6311.) Desired sequence modifications useful in obtaining the desired portions of the ricin A sequence or appended sequences for the construction of expression vectors may be made using site-specific mutagenesis in a manner analogous to that described for the construction of expression vectors below.

The cDNA insert in pRA123, which contained the coding sequence for the entire ricin A chain, was modified by primer directed mutagenesis to place a HindIII site in front of a newly constructed ATG start codon preceding the RTA sequence, and to place a stop signal at the C-terminus. The properly terminating coding sequence for the ricin A chain could then be removed as a HindIII/BamHI cassette and ligated into appropriate transfer vectors.

B.2.a. Cloning of the Ricin B Coding Sequence

The approach followed to obtain recombinant ricin B has been described in European Application No. 86301227.4 (*supra*) and PCTWO/8800593 (*supra*). It is, briefly, as described below.

The sequence published for naturally-occurring ricin B showed the presence of the amino acid sequence, Trp-Met-Phe-Lys-Asn-Asp-Gly, which is associated with minimal codon redundancy. A mixture of all oligonucleotide sequences encoding this sequence was constructed as a probe.

A cDNA library was constructed by isolating mRNA from castor bean seeds, and preparing the corrresponding cDNA by, in general, conventional methods. However, during the construction, appropriate linkers were ligated to the ends of the cDNA so as to obtain inserts bounded by EcoRI/SalI sites. EcoRI/SalI inserts were then ligated into the cloning vector, pUC13, and transformed into *E. coli*. Successful transformants capable of hybridizing with the probe were selected and sequenced.

Colonies were obtained which contained large portions of the ricin B and agglutinin B sequences. In addition, a colony was obtained which contained the sequences for a portion of the putative peptide precursor of both RCA and ricin which was thus shown to contain a twelve amino acid bridging peptide. The cDNA insert contained a sequence which began in the A portion and overlapped into the B region of each. The plasmids derived from the foregoing colonies are designated pRTB5, pRTB4, and pRTA115, respectively.

The cDNA insert in pRTB5, which contained the coding sequence for the entire ricin B chain except for the 11 N-terminal amino acids, was excised and placed in the correct orientation with respect to the lac promoter by insertion into pUC8, to give pRTB151. pRTB151 was modified by the procedure described in Section B.2.b. below to add the appropriate coding sequences, a start codon, and a conveniently placed upstream HindIII site to give pRTB601. The cloning vector used to obtain the cDNA library contains a HindIII site immediaely downstream of the SalI site used for ligation into the vector, and thus the entire coding sequnce including the start codon can be excised by treatment of the modified vector with HindIII.

B.2.b. Construction of the Ricin B Coding Sequence as a HindIII-Cassette - pRTB601

Ten μg of pRTB151 DNA was digested to completion with EcoRI, dissolved in 60 μl S1 buffer and digested for four minutes at room temperature under conditions which remove about 1 base pair of duplex DNA per minute. DNA recovered from the foregoing buffer was dissolved in 60 μl exonuclease III buffer and digested for four minutes at room temperature. Subsequent analysis showed that the plasmid DNA had lost approximately 120 bp from each 3' end, leaving 5' ends available for hybridization. DNA recovered from the exonuclease III buffer was dissolved in 50 μl water and 20 μl used in the ligation/repair reaction below.

Thus, 20 μl sample (2 pmoles) was mixed with 20 pmoles each of the synthetic oligonucleotides:

```
                      Oligo 2
5'-GACCATGATAAGCTTATGGCTGATGTTTGTATGGATCC and
                 HindIII       3'TACCTAGGACTCGGGTATCACGCATAGCATCC-5'
                                                Oligo 1
```

which have complementary sequnces as shown, and wherein Oligo-2 encodes a HindIII site upstream of an ATG start codon. The 5' end of Oligo-1 is complementary to 15 bases at the 5' end of the pRTB151 cDNA sequence as there shown and is complementary to the contiguous missing codons of the ricin B sequence. The 5' end of Oligo-2 is complementary to the 5' sticky end of the vector residue of the exonuclease III treated pRTB151.

The mixture was heated to 60°C for five minutes in order to denature completely completion of single-stranded DNA, cooled to 37°C for five minutes to hybridized complementary strands, and then chilled on ice. The solution brought to polymerase I (Klenow) buffer conditions and reacted for two hours at 12°C in the presence of the 50 μM each of the 4 dNTPs, 0.1 mM NAD, 0.3 units/μl Klenow, and 0.08 units/μl _E. coli_ DNA ligase. The ligation mixture was used directly to transform competent _E. coli_ MM294 and several thousand Amp^R colonies found. Several hundred of these were replicated and grown on nitrocellulose filters and subjected to standard colony hybridization using P^{32} kinased Oligo-2 as probe. Two clones which hybridized with the probe were analyzed by restriction analysis and sequenced, and a correct construction designated pRTB601. pRTB601 thus contains the ricin B coding sequence as a _Hind_III cassette. The upstream _Hind_III site is introduced immediately upstream of the ATG codon in Oligo-2; the downstream _Hind_III site arises from the pUC8 vector plasmid.

B.2.c. Identification of Galactose Binding Sites on Ricin B - Muteins of Ricin B

Ricin B has two functional characteristics, it first plays a role in binding to galactoside on the surface of cells and then participates in the internalization of ricin toxin A chain into the cell. The muteins of ricin B according to the invention have amino acid sequences that are specifically altered from those described sequences herein for ricin toxin B chain. The alterations are made in amino acids that comprise the galactoside binding sites of ricin B, and most preferably in amino acids that affect the binding of ricin B chain to galactosides, e.g., lactose. The muteins of ricin B of the present invention are altered in these amino acids to decrease the binding of ricin B to galactoside.

B.2.d. Identification of Galactoside Binding Sites of Ricin B

B.2.d.1. General Description of the Structure of Ricin B

The three dimensional structure of the ricin B molecule has been determined to a resolution of 2.8 Angstroms A by Robertus et al., and a two dimensional representation of the ricin B chain structure is shown in Figure 1.

The representation shows two domains within the B chain of ricin and each domain has a galactoside binding region. Each domain of the ricin B chain has two disulfide loops, and each domain has a single galactoside binding site. The two domains have folding patterns that are similar and can be classified for purposes of the invention as an amino terminal domain encompassing amino acid residues 1-135 which includes the amino galactoside binding site, and a carboxyl terminal domain encompassing amino acids 136-267 which includes the carboxyl galactoside binding site.

The amino galactoside binding site is defined by two regions of the amino acid sequences, residues 22-28 (Asn22, Val23, Arg24, Asp25, Gly26, Arg27, and Phe28) and residues 35-46 (Gln35 Leu36 Trp37 Pro38 Cys39 Lys40 Ser41 Asn42 Thr43, Asp44 Ala45 and Asn46). Amino acids that can potentially interact with galactose are contained within the residues 22-28 and 35-46. The carboxyl galactoside binding site may be defined by three regions of amino acid sequences, residues 197-200 (Arg197, Glu198, Thr199 and Val200) residues 233-239 (Leu233 Asp234 Val235 Arg236 Ala238 Ser238 Asp239) and residues 244-256 (Gln244, Ile245, Ile246, Leu247, Tyr248, Pro249, Leu250, His251, Gly252, Asp253, Pro254, Asn255 and Gln256). Amino acids that can potentially interact with galactose are contained within the residues 233-239 and 244-256. Not all of the residues described above however are considered to bind or contact to lactose.

A computer analysis of the 2.8 Angstrom (A) crystallographic structure of the ricin B chain was carried out using Mogli protein modeling program on an Evans and Sutherland Graphics System both of which are commercially available from Evans and Sutherland, Salt Lake City, Utah, U.S.A. Table 1, generated using this system, shows the distances in Angstroms of particular amino acid residues of the amino galactoside binding site of ricin B to some part of the lactose residue bound therein.

Table 1

Protein Residue	Residue Atom	Lactose Residue	Lactose Atom	Distance (Angstrom)
Trp37	N	Gal	06	5.95
Trp37	C6	Gal	06	4.47
Trp37	C7	Gal	02	6.67
Arg27	C5	Gal	06	8.56
Phe28	C4	Gal	06	10.9
Pro38	C6	Gal	06	8.93
Val23	C5	Gal	06	8.20
Asn42	0	Gal	03	11.0
Gln35	N	Gal	06	2.09
Gln35	0	Gal	06	2.31
Asn46	N	Gal	04	3.12
Asn46	0	Gal	03	2.45
Asn46	N	Gal	03	2.85
Asp22	0	Gal	03	2.65
Asp22	0	Gal	04	4.21
Asp25	0	Glc	02	3.79
Asp25	0	Gal	03	5.71
Asp25	C3	Gal	06	5.03
Asp25	0	Gal	06	4.26
Asp25	0	Gal	06	5.14
Lys40	N	Gal	03	2.37
Lys40	N	Gal	02	2.86
Arg24	C5	Gal	06	2.86
Asp44	0	Glc	C6	5.29
Asp44	0	Glc	06	5.29

From these data, residues within 3 Angstroms of an atom in lactose are:

From these data, residues within 3 Angstroms of an atom in lactose are:

Asp22 Arg24 Asn46 Gln35 Lys40

Further analysis was carried out to determine the part of the structures of the amino acids of the amino galactoside binding site and lactose bound within the site that fall within specified distances of one another. The results of the analysis, in Table 2, showed that the indicated parts of the following amino acid side chains were within the specified distance of some part of galactose.

Table 2

	Distance		
Residue	3 A	4 A	5A
Asp22	C-COO	C-COO	C-COO
Arg24	C-C-C	C-C-C C=O	ALL SIDE CHAIN
Asp25	C C=O	C-C-COO C=O	C-C-COO C=O
Gly26			
Gln35	C-CON	C-C-CON	C-C-CON
Lys40	C-N	C-C-N	C-C-C-C-N
Asn46	C-N	C-CON	C-CON
Trp37		C3-C8 C11 N	ALL SIDE CHAIN
Asp44			COO

The same computer analysis was carried out on the carboxyl galactoside binding site. Table 3 shows the distances of particular amino acids residues of the carboxyl galactoside binding site of ricin B to some part of the lactose residue bound therein.

Table 3

Carboxyl Terminal Domain

Protein Residue	Atom	Lactose Residue	Atom	Distance-(Angstrom)
Tyr248	0	Gal	02	5.34
Try248	C6	Gal	02	6.42
Tyr248	C8	Gal	03	4.61
Tyn248	C5	Gal	03	6.43
Arg236	N	Glc	02	8.09
His251	C6	Gal	03	2.97
Asp234	0	Gal	04	3.21
Asp234	0	Gal	04	3.30
Asp255	N	Gal	04	2.58
Asn255	0	Gal	04	3.74
Asn255	0	Gal	03	4.06
Asn255	N	Gal	03	4.39
Ala237	0	Gal	03	3.51
Ile246	C6	Gal	C6	2.94

From these data, residues within 3 Angstroms of an atom in lactose are:

Ile246 Asn255 His251

The same analysis used to generate the data in Table 2 showed that the indicated parts of the structure amino acid residues of the carboxyl galactoside binding site of Table 4 are within the specified distances of a part of galactose.

Table 4

	Distance		
Residue	3 A	4 A	5 A
His251	N-C-N	C-N-C-N-C	ALL SIDE CHAIN
Asn255	C-N	C-C-N	ALL SIDE CHAIN
Arg236	Ca-C	C-C-C C=0	C-C-C C=0
Ile246	C-C	C-C C-C	C-C C-C
Asp234	C-C00	C-C00	
Ala237	C C=0	C C=0	
Gln256		C-N	
Tyr248	C-C-C-C-C ALL SIDE CHAIN		

B.2.e. Alterations in Ricin B Amino Acids Involved in Bonding to Galactoside

B.2.e.1. Alteration of Hydrogen Bonding Amino Acids

All of the amino acids in Tables 2 and 4 have proximities close enough to galactose to be involved in the binding of galactoside in the respective amino or the carboxy galactoside binding sites.

Asp22, Gln35, Lys40, and Asn46 all are within 3.25 A of at least one atom of galactose. The nature of the side groups of the amino acids and galactose that are within 3.75 A of one another suggest that they are hydrogen bonded. The approximate bond lengths of biologically important hydrogen bonds range from to 3.10± 0.13 A and below. (See Molecular Biology of the Gene, Watson ed., W. A. Benjamin Inc., New York, 2nd Edition (1970). A hydrogen bond can be considered to be an intermediate stage of transfer of a proton from an acid to a base. The strength of a hydrogen bond increases with the acidity (ability to donate a proton) of the proton donor and with the basicity (ability to accept a proton) of the proton acceptor. Hydrogen bonds can arise between covalently bound hydrogen atoms having a positive charge and negatively charged covalently bound, acceptor atoms, e.g., $C=O^-$, or between groups of atoms having a unit charge. By changing the charge of the side groups of the amino acids forming hydrogen bonds, or by chemical derivatization of the side group involved, or by substitution of the amino acid with a different amino acid that does not form a hydrogen bond, for example, one that has uncharged or oppositely charge side groups, or lastly, by deleting the amino acid involved in hydrogen bonding, the binding of galactoside by the galactose binding site is decreased.

Within the scope of the invention are alterations in amino acids that form hydrogen bonds with the galactoside. Such alterations include amino acid derivatives, amino acid substitutions and deletions that result in a decrease in binding of galactoside to the galactoside binding site or sites. Amino acid residues to which such alterations may be carried out are those that form hydrogen bonds with the galactoside, and amino acids stabilizing amino acids that form hydrogen bonds with the galactoside. With respect to the amino galactoside bonding site, residues Asp22, Arg24, Gln35, Lys40 and Asn46 of the ricin B chain are in positions that indicate potential formation of hydrogen bonds with galactoside. With respect to the carboxyl galactoside bonding site, residues His251 and Asn255 are in positions that indicate potential formation of hydrogen bonds with galactoside. Furthermore, Asp234 also is in a position that suggests hydrogen bond formation.

Amino acid residues that stabilize an amino acid which is in a position to form a hydrogen bond with galactoside are Asp22 of the amino galactoside binding site, and Asp234 of the carboxy galactoside binding site. Both of these residues may also participate in hydrogen bonding to the galactoside. Intervening water molecules between amino acid residues, such as Asn255, may hydrogen bond to galactose.

Muteins of ricin B according to the invention may be formed by deletion or substitution of at least one of the amino acids that form hydrogen bonds with galactoside or stabilize amino acids that form hydrogen bonds with galactoside. Such substituting amino acids that do not form hydrogen bonds will generally have either a side group that lacks charge, such as glycine, alanine, valine, isoleucine, leucine. Substituting amino acids with no side chain (glycine) or short side chain are generally preferred. Also preferred are amino acids having side chains that are oppositely charged from side chain of the amino acid for which it substitutes. Thus, when aspartic acid or glutamic acid are the residues in the native ricin B chain to be substituted, both of which have negatively charged carboxyl side groups, lysine and arginine, which have terminal amino side groups are preferred in the ricin B mutein. Conversely when lysine or arginine are the residues in the native ricin B chain to be replaced, aspartic or glutamic acid residues are used as replacements in the ricin B chain mutein. In general, it is preferred to replace hydrogen bonding or stabilizing amino acids with those having small uncharged side groups, such as glycine and alanine. Such amino acids are sufficiently small that binding of the galactoside through the weak interaction of Van der Waals forces is not expected.

The following Tables 5 and 6 show the substitutions for particular residues in decending order of preference. The most preferred are at the top of the list and the least preferred substitutions are at the bottom of the list. Amino acids in the middle of the list are placed only in approximate relative preference. Each substitution may be made singly independent of substitution of any of the other replaced amino acids. Multiple amino acids in the native sequence may be replaced.

Table 5

Amino Galactoside Binding Site

Asp22	Arg24	Asp25	Gln35	Trp37	Lys40	Asn46
Gly	Gly	Gly	Gly	Gly	Gly	Gly
Ala	Ala	Ala	Ala	Ala	Ala	Ala
Ser	Asp	Ser	Asp	Ser	Asp	Lys
Lys	Glu	Lys	Glu	Thr	Glu	Arg
Arg	Val	Arg	Asn	Asn	Val	Asp
Asn	Ser	Asn	Leu	Val	Ser	Glu
Glu	Thr	Glu	Ile	Gln	Thr	Gln
Gln	His	Gln	Val	Cys	His	Leu
Leu	Lys	Leu	Lys	Lys	Arg	Ile
Ile	Cys	Ile	Arg	Arg	Cys	Val
Val	Asn	Val	His	Asp	Asn	His

Table 6

Carboxyl Galactoside Binding Site

Asp234	Arg236	Ala237	Ile246	Tyr248	His251	Asn255
Gly	Gly	Leu	Gly	Gly	Gly	Gly
Ala	Ala	Ile	Ala	Ala	Ala	Ala
Ser	Asp	Val	Ser	Ser	Asp	Lys
Lys	Glu	Lys	Thr	Thr	Glu	Arg
Arg	Lys	Arg	Val	Asp	Lys	Asp
Asn	His	Asp	Asn	Glu	Arg	Gln
Glu	Ser	Glu	Gln	Asn	Ser	Glu
Gln	Thr	Ser	Cys	Gln	Thr	Leu
Leu	Val	Thr	Lys	Val	Val	Ile
Ile	Leu	Asn	Arg	Ile	Leu	Val
Val	Ile	Gln	Asp	Leu	Ile	His
His	Phe	Cys	Glu	Lys	Phe	Ser
Thr	Tyr	Met	Ile	Arg	Tyr	Thr
Met	Gln	Gly	Leu	His	Gln	Met
Cys	Asn	His	Met	Met	Asn	Cys
Phe	Met	Phe	His	Cys	Met	Phe
Tyr	Cys	Tyr	Phe	Trp	Cys	Tyr
Trp	Trp	Trp	Tyr	Phe	Trp	Trp
Pro	Pro	Pro	Pro	Pro	Pro	Pro

B.2.e.2. Alteration of Aromatic Amino Acids

Both the amino and carboxyl galactoside binding sites have one site that is formed by an aromatic amino acid residue, Trp37 and Tyr248 in the amino and carboxyl galactoside binding sites, respectively. The positioning of the aromatic side chain of these two amino acids in the respective galactoside binding site is substantially parallel to the ring of the lactose moiety in the 2.8 A resolution crystal structure of ricin B. As set out in Tables 2 and 4, the aromatic side chain of both of these amino acids is about 5 A from the lactose residue and substantial portions of each amino acid are within 4 A of the lactose residue. The distances of the side chains from the lactose ring are such that strong nonspecific attractive forces or Van der Waals interactions are indicated. Van der Waals interactions may occur over distances such as those indicated above between the aromatic ring of the amino acids and the ring structure of lactose.

In the muteins of ricin B, according to the invention, the aromatic amino acids are deleted or are substituted with amino acids that do not lead to stabilization of nonspecific attractive forces such as Van der Waals invention. In general, substitutions will be made with amino acids that do not have aromatic or heterocyclic side chains. Thus, substitution with tryptophan, phenylalanine, tyrosine and histidine are not desirable for either Trp37 or Tyr248. The substitutions are preferably made with amino acids that have small side chains. Most preferred are those that do not have significant charge separation and therefore do not have the potential for formation of stabilizing hydrogen bonds. Glycine and alanine are particularily preferred. Not desirable are large uncharged side chains such as those of leucine and isoleucine which, because of their extended uncharged structure, may have sufficient proximity to the lactose residue to stabilize the bonding thereto by Van der Waals interaction.

B.2.e.3. Double Alterations: Substitutions

Of course, combinations of substitutions at more than one residue of either or both galactoside binding sites are within the scope of the invention. To decrease binding to galactose by both the amino and carboxyl galactoside binding sites, it is preferable to alter at least one amino acid in each of the galactoside binding sites. Also within the scope of the invention are deletions of more than one residue of either or both galactoside binding site. Furthermore, combinations of at least one substitution and at least one deletion at amino acid residues of either or both galactoside binding site are within the scope of the invention.

The following Tables 7 and 8 list the preferred double substitutions in the amino and carboxy galactoside binding sites. Of course one or both galactoside binding sites may be double substituted or on galatoside binding site may be singly substituted while the other is doubly substituted.

Table 7

Amino Galatoside Binding Site

Site 1	Site 1 Substitution	Site 2	Site 2 Substitution
Asp22	Gly	Asn46	Gly
Asp22	Gly	Asn46	Ala
Asp22	Ala	Asn46	Gly
Asp22	Ala	Asn46	Ala
Asp22	Gly	Trp37	Gly
Asp22	Gly	Trp37	Ala
Asp22	Ala	Trp37	Gly
Asp22	Ala	Trp37	Ala
Gln35	Gly	Trp37	Gly
Gln35	Gly	Trp37	Ala
Gln35	Ala	Trp37	Gly
Gln35	Ala	Trp37	Ala
Asp22	Gly	Asp25	Gly
Asp22	Gly	Asp25	Ala
Asp22	Ala	Asp25	Gly
Asp22	Ala	Asp25	Ala

Table 8

Carboxy Terminal Site

Site 1	Site 1 Substitution	Site 2	Site 2 Substitution
Asp234	Gly	Asn255	Gly
Asp234	Gly	Asn255	Ala
Asp234	Ala	Asn255	Gly
Asp234	Ala	Asn255	Ala
Asn255	Gly	Tyr248	Gly
Asn255	Gly	Tyr248	Ala
Asn255	Ala	Tyr248	Gly
Asn255	Ala	Tyr248	Ala
Asn255	Gly	His251	Gly
Asn255	Gly	His251	Ala
Asn255	Ala	His251	Gly
Asn255	Ala	His251	Ala

The following Tables 9 and 10 indicate substitutions embodying modifications which increase the side chain size on one group and eliminate hydrogen bonding or Van der Waals interactions:

Table 9

Amino Terminal Site

Site 1	Site 1 Substitution	Site 2	Site 2 Substitution
Asp25	Leu	Asn46	Gly
Asn22	Leu	Asn46	Gly

Table 10

Carboxyl Terminal Site:

Site 1	Site 1 Substitution	Site 2	Site 2 Substitution
Asp234	Leu	Asn255	Gly

B.2.e.4. Substitutions with Cysteine

A cysteine residue or residue may be inserted into one or both of the galactoside binding sites of ricin B chain. The thiol group of cysteine reacts quickly under mild conditions with iodoacetate, iodoacetamide, N-ethylmaleimide and other reagents that are specific, or can be made specific, for thiol groups.

By replacing a side chain of an amino acid that contacts galactose in the binding site with cysteine, a site is provided for easy manipulation. After ricin B chain is folded, 8 thiol groups have formed 4 disulfide bonds. The ninth thiol group, Cys4, remains free. This can be either left and chemically modified, or it can be removed by site-specific modification (changed to a serine or other residue). If the molecule being modified is ricin, then Cys4 would be left and it would be linked to the interchain thiol group of ricin A chain to form a disulfide bond. In such a molecule the only thiol that can react with sulfhydryl reagents would be the cysteines inserted into the galactose binding pockets. The cysteine at 171 of ricin A chain has been shown to be unavailable for reaction as it is deeply situated in a hydrophobic region of the molecule.

The substitution of a cysteine residue for an amino acid in either or both of the galactoside binding sites may be sufficient to decrease or eliminate the binding of galactoside to ricin B. In addition to the extent that the binding of galactoside is not decreased by the above-mentioned cysteine substitution, the cysteine residue may be derivatized with thiol specific groups such as alkylating agents to yield a cysteine derivative that interferes with galactoside binding.

The size of the thiol specific reagent may be increased if iodoacetamide, iodoacetate or N-ethylmaleimide did not prevent galactose binding. For example, the carboxyl group of iodoacetate may be linked in an amide bond to glycine. It could link to the amino group of cysteine in which the thiol group was blocked by a disulfide, such as with 5-thio-2-nitrobenzoic acid (TNB). After reaction with the recombinant ricin B chain, the thiol could be exposed by gentle reduction under conditions that did not reduce the disulfides on the protein. The thiol group could be modified with iodoacetate, iodoacetamide or N-ethylmaleimide. Various means for chemically derivatizing the cysteine residue placed in the galactoside binding site are possible and are considered within the scope of the invention to the extent that the ricin B mutein shows decreased binding to galactoside while retaining the ability to aid in translocation of the toxin molecule.

The following residues may be modified by substitution with cysteine:

Amino terminal	Cartoxyl terminal
Asp 22	Asp 234
Arg 24	Arg 246
Asp 25	Ala 237
Gly 26	Ile 246
Gln 35	Tyr 248
Trp 37	His 251
Lys 40	Asn 255
Asn 46	

Asp 22 and Asn 46 for the amino galactoside binding site and Asp 234 and Asn 255 for the carboxyl galactoside binding site are the preferred residues for substitution with cysteine.

The nucleic acid and amino acid sequences in the amino terminal site are shown in Figure 2A.

Residues 22 (Asp) and 46 (Asn) in the amino terminal site can be modified using the following oligonucleotides for site specific modification:

```
5'-CGAAATGGTCTATGTGTTTGCGTTAGGGATGGAAGATTCC-3'        Asp 22 to Cys
   CGAAATGGTCTATGTGTTGATGTTAGGGATGGAAGATTCC           ricin B sequence
   ArgAsnGlyLeuCysValAspValArgAspGlyArgPheHis
                     22

5'-TGCAAGTCTAATACAGATGCAAATTGCCTCTGGCCATGCAAGTCT-3'   Gln 46 to Cys
   TGCAAGTCTAATACAGATGCAAATCAGCTCTGGCCATGCAAGTCT      ricin B sequence
   CysLysSerAsanThrAspAlaAsnGlnLeuTrpProCysLysSer
                              46
```

The nucleic acid and amino acid sequence of the carboxyl terminal site are shown in Figure 2B.

Amino acids 234 (Asp) and 255 (Asn) in the carboxyl galactoside binding sites can be modified using the following oligonucleotides:

```
5'-GTGGGTTGGTGTTATGCGTGAGGGCATCGGATCC-3'   Asp234 to Cys
   GTGGGTTGGTGTTAGATGTGAGGGCATCGGATCC      ricin B sequence
     G  L  V  L  D  V  R  A  S  D  P
                234

5'-CCTCTCCATGGTGACCCATGCCAAATATGGTTACC-3'  Asn255 to Cys
   CCTCTCCATGGTGACCCAAACCAAATATGGTTACC     ricin B sequence
   P  L  H  G  D  P  N  Q  I  W  L  P
                     255
```

B.2.e.5. Methods for Carrying out Alterations of the Amino and Carboxy Galactoside Binding Sites of Ricin B

A number of known methods may be used to carry out the desired modifications to form the ricin B muteins according to the invention.

Ricin B protein having the desired amino acid replacement or deletion may be made by conventional Merrifield synthesis as is known in the art. However, Merrifield synthesis of a complete ricin B molecule is undesirably complicated.

Substitutions and deletions may be accomplished by digesting to completion DNA encoding the native ricin B protein with specified endonucleases that cut in the region of the DNA surrounding the amino acid to be altered, removing the DNA fragment which encodes the amino acid residue or residues of the native ricin B galactoside binding site to be altered, and ligating, either under blunt ended or sticky ended conditions as appropriate, a double stranded DNA made of complementary chemically synthesized oligonucleotides that encode the desired amino acid alteration. The means for making such oligonucleotides are known and include commercially available automated DNA synthesizers such as that made by Biosearch, San Rafael, California.

Site-specific mutagenesis may also be used to carry out alterations to the DNA encoding specific amino acid. In general, the DNA encoding ricin B chain in the region to be altered is cut using an appropriate endonuclease, the fragment carrying the specific ricin region is removed, ligated into an appropriate vector such as an M13 vector and is mutagenized using a single-stranded oligodeoxyribonucleotide primer synthesized to insert, change, or delete nucleotides from the fragment after replication with an appropriate DNA polymerase.

To obtain the DNA fragment encoding the desired ricin B region, endonuclease restriction sites that are found in the native ricin B chain sequence may be used, or unique restriction sites on either side of the areas of interest are made in the DNA sequence of ricin B.

In a preferred embodiment, a new site for cleavage by SalI in the area of the amino galactoside binding site is made using site-specific mutagenesis to modify the sequence at Val21 and Asp22. Another site is created for cleavage by PvuII using the same technique to modify the base sequences around Gln47 and Leu48. Both modifications may be made without changing the amino acid sequence of ricin B. Figure 2A illustrates the position of the SalI and PvuII sites that can be created and the oligonucleotide sequences that can be used to mutagenize the sequence of ricin B and retain the amino acid sequence.

Also in a preferred embodiment, a new site for cleavage by XbaI in the area of the carboxyl galactoside binding site is constructed by site-specific mutagenesis by modifying the sequence at Val232, Leu233 and Asp234. Another unique site is created for cleavage downstream of the carboxyl terminal galactoside binding site of the ricin B chain by SacII. Figure 2B illustrates the position of the XbaI and SacII sites, and the oligonucleotide sequences that are used to mutagenize the sequence of ricin B and retain the amino acid sequence.

The unique restriction sites introduced into the ricin B sequence are produced by site-specific mutagenesis using conventional means. The above mentioned restriction sites are preferred because they do not alter the amino acid sequence of ricin B. Other unique restriction sites may be introduced as long as the amino acid sequence of ricin B is not changed, or if changed, the new sequence does not affect the essential biological properties of ricin B that relate to translocation.

As mentioned above, various methods may be used to introduce changes in the DNA sequence encoding amino acids of the amino terminal and carboxyl terminal galactoside binding sites. Double stranded oligodeoxyribonucleotides having "sticky ends" compatable with the unique restriction site engineered into the ricin B sequence by site-specific mutagenesis may be used. Such oligonucleotides may be made by conventional commercially available oligonucleotide synthesizers.

Table 11 shows the double-stranded oligodeoxyribonucleotide spanning the unique SalI to PvuII site engineered into the amino terminal region of the ricin B chain that encompasses the amino galactoside binding site. Table 12 shows the double-stranded oligodeoxyribonucleotide spanning the unique XbaI to SacII sites in the carboxyl region of ricin B chain that encompasses the carboxyl galactoside binding site. Each table shows the nucleotide changes required for the substitution of various amino acids. As mentioned above, the nucleotide change may be made to single or multiple amino acids in this region of the ricin B molecule. In addition, any of the changes may be made independently of all other changes.

Table 11

```
Amino Acid #
                          22                                     35    37       40                46
22  35  37  40  46      TCGACGTTAGGGATGGAAGATTCCACAACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAG
Asp Gln Trp Lys Asn         ACAATCCCTACCTTCTAAGGTGTTGCCTTTGCGTTATGTCAACACCGGTACGTTCAGATTATGTCTACGTTTAGTC
    Gly Gly Gly Gly                                              GGG   GGT      GGC               GGT
                                                                 CCC   CCA      CCG               CCA
    Ala Ala Ala Ala                                              GCG   GCG      GCG               GCG
                                                                 CGC   CGC      CGC               CGC
    Asp Ser Leu Asp                                              GAT   TCC      CTG               GAT
                                                                 CTA   AGG      GAC               CTA
    Glu Thr Ile Gln                                              GAA   ACC      ATC               GAA
                                                                 CTT   TGG      TAG               CTT
    Asn Val Asp Gln                                              AAC   GTG      GAT               CAG
                                                                 TTG   CAC      CTA               GTC
                Leu                                                                               CTG
                                                                                                  GAC
```

Table 12

```
Amino Acid #
                   234                                       248      251         255
234 248 251 255 CTAGATGTGAGGGCATCGGATCCGAGCCTTAAACAAATCATTCTTTACCCTCTCCATGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACCGC
Asp Tyr His Asn     TACACTCCCGTAGCCTAGGCTCGGAATTTGTTTAGTAAGAAATGGGAGAGGTACCACTGGGTTTGGTTTATACCAATGGTAATAAAACTATCTGG
Gly Gly Gly Gly    GGT                                       GGT      GGT         GGT
                    CA                                       CCA      CCA         CCA
Ala Ala Ala Ala    GCG                                       GCG      GCG         GCG
                    GC                                       CGC      CGC         CGC
Gly Ser Asp Glu    GAA                                       TCC      GAT         GAA
                    TT                                       AGG      CTA         CTT
        Glu Gln                                                       GAA         CAG
                                                                      CTT         GTC
        Lys Asp                                                       AAA         GAT
                                                                           TTT         CTA
```

Deletions of amino acids may be made using essentially the same method, however, instead of changing the nucleotide sequence to encode a substituted amino acid, the complete trinucleotide codon encoding the amino acid to be deleted is removed. Such deletions are particularly desirable if they do not change the comformation of the protein, though not necessarily preferred for Trp37 and Tyr248.

Modifications of the DNA sequence encodinq Asp22 may be made using the double-stranded break and repair method of Mandecki, Proc. Natl. Acad. Sci. USA 87:7177-7181 (1986).

Briefly, SalI cleaves the sequence 5'GTCGAC-3' at a position immediately 3' of the 5' G of this sequence, and the GAC portion codes for Asp22 in ricin B chain. Briefly, the plasmid comprising the ricin B sequence is cleaved at the SalI site, inserted as described above, to convert the circular structure to a linear one. An oligonucleotide containing sequences at either end that are identical to the ricin B chain DNA sequence with the site at residue 22 mutated to the desired amino acid is mixed with the linear plasmid. After heating and annealing, the DNA is used to transform cells rendered competent by calcium chloride treatment and incubation on ice followed by incubation at 37C for a short time. Transformed cells containing the desired mutation are then detected by using ^{32}P-labelled oligonucleotide probes.

The sequence flanking and including the SalI site is as follows (the gap is to illustrate the SalI site):

```
                                                SalI cleavage site
5'                                                Asp22
  CCCATAGTGCGTATCGTAGGTCGAAATGGTCTATGTG         TCGACGTTAGGGATGGAA
  GGGTATCACGCATAGCATCCAGCTTTACCAGATACACAGCT         GCAATCCCTACCTT
3'

                                                                        3'
GATTCCACAACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAG
CTAAGGTGTTGCCTTTGCGTTATGTCAACACCGGTACGTTCAGATTATGTCTACGTTTAGTC
                                                                        5'
```

The oligonucleotides to be used to repair the strand break and insert new amino acids substituting for Asp22 are shown in Table 13.

Modifications to the DNA sequence of the carboxyl galactoside binding site encoding Asp234 are made in essentially the same manner as described for the modifications of Asn22.

The sequence flanking and including the <u>XbaI</u> site is as follows (the gap is to illustrate the <u>XbaI</u> site):

```
5'                                       XbaI cleavage site
GGAACCATTTTAAATTTGTATAGTGGGTTGGTT        CTAGATGTGAGGGCATCGGATCCG
CCTTGGTAAAATTTAAACATATCACCCAACCAAGATC        TACACTCCGGTAGCCTAGGC
3'

                                                    3'
AGCCTTAAACAAATCATTCTTTACCCTCTCCATGGTGACCCAAACCAAATATG
TCGGAATTTGTTTAGTAAGAAATGGGAGAGGTACCACTGGGTTTGGTTTATAC
                                                    5'
```

Gly, Ala and Glu codons are accomodated with retention of the <u>XbaI</u> restriction sequence as shown above in connection with Table 13. The repair oligonucleotides to introduce codons for amino acids that change the <u>XbaI</u> restriction sequence for Gln and Asn are shown in Table 14.

Table 13

Amino Acid #			
22		22	
Asp	GCGTATCGTAGGTCGAAATGGTCTATGTGTC	GAC	GTTAGGGATGGAAGATTCGACAACAACGG
Gly		GGT	
Ala		GCG	
Asn		AAC	
Glu		GAA	
Gln		CAG	

Table 14

Amino Acid #			
234		234	
Asp	CCATTTTAAATTTGTATAGTGGGTTGGTTCTA	GAT	GTGAGGGCATCGGATCCGAGCC
Gln		CAG	
Asn		AAC	

In general, cloning and expression vectors used in conjunction with the ricin B sequence into which the unique restriction sites have been introduced, will lack the unique restriction sites. If necessary, restriction sites in the vector that are the same as the unique restriction sites may be removed by site-specific mutagenesis as is known in the art.

B.3. Cloning of Ricin B isotoxins D, and E and muteins thereof

As mentioned above, there are several known isotoxins of ricin B and these include ricins D, and E. Furthermore, ricin E has recently been shown to consist of at least two molecular species, E1 and E2. It will be appreciated by those skilled in the art that cloning of these molecules is achievable using the materials and methods described in the preceding sections that permit cloning of ricin B. It will also be appreciated by those skilled in the art that muteins of the isotoxins having reduced galactose binding are realizable, using the materials and methods shown for constructing muteins of ricin B, because the amino acid sequences of the isotoxins are, to a large degree, similar to ricin B. This is particularly true of the amino acids that compose the galactose binding regions. Butterworth, R. and Lord, M. Dur. J. Biochem. 137, 57 (1983), Halling et al., Nucleic Acid Res., 13, 8019 (1985) O'Hare, et al., FEB. 216, 73 (1987), and Araki, T. and Funatsu, G., Bioochemica et Biophysica Acta 911, 191 (1987).

Thus, the isotoxins D and E and muteins thereof are capable of being cloned and expressed by the instant invention.

B.4. Cloning of Full-Length Ricin and RCA Encoding Clones

The full-length sequences encoding ricin D, putative ricin E, and RCA in precursor form were obtained, using the messenger RNA prepared as described above for ricin A, to obtain a cDNA library, and then probing the library to retrieve the desired cDNA inserts. The library was prepared using the method of Okayama and Berg (Mol. and Cell Biol. (1983) 3:230-289) and was probed using the same 35-mer used for ricin A-encoding sequences. Out of several thousand transformants with cloning vector, a number of positively hybridizing clones were obtained.

Positively hybridizing colonies were subjected to restriction analysis and showed restriction patterns corresponding to ricin D and to RCA, and a third type which corresponded to neither. The cDNA inserts from representative clones of each of the three types were sequenced. The results of the sequence information for ricin D are shown in Figure 3. The sequence for ricin D is used in the construction of the transfer vectors described below. However, related sequences such as those cross-hybridizing to the probe may also be used in the transfer vectors disclosed. As stated for ricin A above, the procedures set forth herein to isolate the sequences need not be repeated, as synthetic methods are available so that the DNA sequences shown in the figures can be constructed using chemical and enzymatic means in vitro.

In addition, the DNA sequence coding for a plant toxin of the ricin type has been published. (Lamb, F.I. et al., supra; Lord, J.M. et al., supra).

The inserts described above can be placed into expression vectors in a manner analogous to that described for ricin A. For the straightforward expression of the coding sequences contained in the isolated inserts, the inserts are subcloned into M13 vectors for site-directed mutagenesis to place an ATG start codon and a HindIII site at the beginning of the mature protein, in a manner analogous to that set forth for ricin A above, or to place a HindIII site immediately prior to the ATG of the secretory leader sequence where appropriate. The mutagenized DNAs can be retrieved from the M13 vectors by cleaving with PstI, blunt-ending with Klenow, digestion with HindIII at the newly created site, and isolation of the appropriate length sequence.

To facilitate conversion of the precursors to either RCA or the ricin toxins, modifications may be made, in particular in the linker portion, to provide suitable means for detaching the A and B portions. A variety of strategies are possible. Two convenient ones are: 1) construction of a trypsin cleavage site by creating an "arg-arg" form of the linker wherein the proline following the arginine residue already present is replaced by another arginine; and 2) insertion of a stop and a start codon in the linker region so that the A and B regions are separately but simultaneously produced.

C. Methods Employed

C.1. Transformations and Transfections

Transformation of E. coli cells was done according to procedures set forth in T. Maniatis, E.F. Fritsch and J. Sambrook Molecular Cloning: A Laboratory Manual (1982) Cold Spring Harbor Press.

Transfections of Sf9 Spodoptera frugiperda cells are accomplished using a modification of the calcium phosphate precipitation technique (Graham, F.L. et al., 1973, Virology 52:456) as modified for insect cells (Burand, J.P. et al. (1980) Virol., 101:286; Carstens, E.B. et al. (1980) Virol., 101:311) and further described by Summers, M.D. and Smith, G.E. (A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas A&M Press, 1987).

C.2. Vector Construction

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art and are described in Maniatis, T. et al., supra. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

Site specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 μg of plasmid or DNA sequence is cleaved by one unit of enzyme in about 20 µl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction, and the nucleic acid recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Methods in Enzymology (1980) 65:499-560.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 minutes at 20 to 25°C in 50 mM Tris pH 7.6, 5 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and about 50 µM of each dNTP. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. If desired, selective repair can be performed by supplying only one of the, or selected, dNTPs within the limitations dictated by the nature of the sticky ends. After treatment with Klenow, the mixture is extracted with pehnol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single-stranded portion.

Synthetic oligonucleotides may be prepared by the triester method of Matteucci, et al., J. Am. Chem. Soc. (1981) 103:3185-3191 or using automated synthesis methods. Kinasing of single strands prior to annealing or for labeling is achieved using an excess, e.g., approximately 10 units of polynucleotide kinase to 1 nM substrate in the presence of 50 mM Tris, pH 7.6, 5 mM $MgCl_2$, 10 mM DTT, 1-2 mM ATP. If kinasing is for labeling or probe, the ATP will contain high specific activity 32γP.

Ligations are performed in 15-30 µl volumes under the following standard conditions and temperature: 50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 10 mM DTT, 100 µg/ml BSA, 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C. Ligations are usually performed at 33-100 µg/ml total DNA concentrations (5-100 nM total end concentration).

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) in order to remove the 5' phosphate and prevent religation of the vector. BAP digestions are conducted at pH 8.5 in approximately 50 mM Tris, 5mM $MgCl_2$, using 0.1-1 unit of BAP per µg of vector at 37-55°C for about one hour. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

C.3. Modification of DNA Sequences

For portions of vectors derived from cDNA or genomic DNA which require sequence modifications, site specific primer directed mutagenesis is used. This technique is now standard in the art, and is conducted using a synthetic oligonucleotide primer complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the orignal strand are sufficient to prevent hybridization. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered. Details of site specific mutation procedures are described below in specific examples.

C.4. Verification of Construction

In the constructions set forth below, correct ligations for plasmid construction are confirmed by first transforming E. coli strain MM294, or other suitable host with the ligation mixture. Miniprep DNA was prepared according to Ish-Horowicz, D. et al. (Nucl. Acids Res. (1981) 9:2989) and screened by restriction analysis. DNA may be further analyzed by restriction and/or sequenced by the dideoxy method of Sanger, F., et al., Proc. Natl. Acad. Sci. (USA) (1977) 74:5463 as further described by Messing, et al., Nucleic Acids Res. (1981) 9:309, or by the method of Maxam, et al., Methods in Enzymology (1980) 65:499.

C.5. Transfer Vector Construction

Because the genome of AcNPV is so large (125kb), there are too many restriction sites to allow site-specific insertion of heterologous genes. Therefore, it is necessary to derive recombinant virus, containing the gene to be expressed, through homologous recombination between viral DNA and genetically engineered chimeric plasmids called transfer vectors.

The transfer vectors which have been described by Smith, G.E., et al. ((1983), supra) were originally constructed by cloning the AcNPV EcoRI-1 fragment containing the polyhedrin gene into the EcoRI site of E. coli plasmid pUC8 (Vieira, J., et al., Gene 19:259-268 (1982)). A series of plasmids or transfer vectors having single BamHI cloning sites at various locations in the polyhedrin gene were created as described (Smith et al., 1983, supra). One of these, pAc373, has a single BamHI site 50 bp downstream from the polyhedrin cap site i.e., 8 bp before the polyhedrin ATG translation initiation codon (Smith et al., (1985) supra). The transfer vectors, pAc610 and pAc611 have the polylinker from M13mp10 and M13mp11, respectively, inserted at this BamHI site of pAc373 (Summers, M.D. et al., personal communication). Partial nucleotide sequence of pAc401 and pAc436 transfer vectors for the production of polyhedrin/foreign gene fusion proteins is also reported (Summers, M.D. et al., 1987, p. 53, supra).

C.6. Preparation and Isolation of Recombinant AcNPV Virus

Detailed methods for the generation of recombinant virus can be found in European Patent Application No. 0127839 to G.E. Smith and M.D. Summers of the Texas A & M University System published December 12, 1984. In general, 2 µg of genetically engineered transfer vector DNA and 1 µg of AcNPV viral DNA are cotransfected onto monolayer culture cells of Spodoptera frugiperda. The infected cells usually show viral occlusions by day 3 or 4, with 10-90% of the cells being infected. The virus titer of the medium is expected to be 10^7 pfu/ml and 0.1%-0.5% are expected to be recombinant virus.

Several methods for the detection of recombinant virus are known in the art. Visual detection of the plaques is best achieved using a low power dissecting microscope and observing the plaques on inverted plates with a black background and illumination from the side. More unequivocal methods for detecting recombinants are plaque hybridization using DNA probes to the cloned gene. Antibody probes to the product of the cloned gene may also be employed.

Isolation of the recombinant virus is achieved through plaque purification of serially infected monolayer cells overlayed with soft agar. After two or three cycles the recombinant virus would be seen as separate plaques showing the characteristic occlusion-negative morphology. The plaques, containing about 10,000 pfu of virus, are picked using a sterile Pasture pipet and transferred to 2 ml of medium.

C.7. Insect Cell Culture

Methods for insect cell cultures are well known in the art and detailed procedures for their cultivation can be found in Summers, M.D. et al. (1987, supra) or in EPO 127,839 to G.E. Smith et al. The insect expression host of the current invention, Spodoptera frugiperda (Sf9) is well suited to the production of heterologous proteins because of its ability to grow in either monolayer or suspension culture.

As monolayer cultures, Spodoptera frugiperda cells will divide every 18-24 hours depending on the culture media. The cells do not require carbon dioxide to maintain the pH of the medium and they will grow well at temperatures between 25-30°C. Subculturing is done 2 or 3 times a week when the cells are confluent. Because insect cells are loosely adherent they are easily resuspended without the need of proteases.

Suspension culture conditions will vary depending on the medium and culture volume and should be determined empirically. Subculturing is required when the cell density reaches 2×10^6 cells/ml by replacing 80% or more of the culture with an equal volume of fresh medium. With suspension cultures larger than 500 ml it becomes necessary to aerate by either bubbling or diffusion.

Preferred media and culture conditions can be found in Summers, M.D. et al., A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experiment Station Bulletin No. 1555.

C.8. Electrophoretic Analysis of Expression Products

In order to concentrate expression products, culture supernatants were incubated with Protein A-Sepharose CL-4B beads cross-linked to antibody to either ricin A (αRTA) or ricin B (αRTB) for 1 hour. The beads were then pelleted and the supernatants reserved. The beads were washed once in 0.5 ml buffer containing 1M LiCl, 20 mM Tris-HCl pH 8.0 and 0.5% NP40, three times in 0.5 ml buffer containing 150 mM NaCl, 20 mM Tris-HCl pH8 and 0.5% NP40 and then suspended in sample loading buffer lacking reducing agent. The samples were then heated to 37°C for 5 minutes, pelleted and the supernatant removed to new tubes These supernatants were adjusted to contain 0.14 M beta-mercaptoethanol or 20 mM DTT, heated to 100°C for 4 min. and run on SDS-PAGE.

SDS-PAGE was performed essentially according to the procedure of Laemmli (Nature (1970) 227:680-685). Immunoblotting procedures have been described in European Publication No. 219,286 published April 22, 1987, essentially as described below.

Immunoblotting of the gel onto nitrocellulose (Schleicher and Schuell, 0.45 μmeter) was performed in a Bio-rad Trans-blot cell at 35 V for one hour at room temperature essentially according to published methods (Towbin et al. (1979) Proc. Natl. Acad. of Sci., USA, 76:4350-4354; Bittner et al. (1980) Anal. Biochem., 102:459-0471; Burnette et al. (1981) Anal. Biochem., 112:195-203). Following transfer, nonspecific antibody binding sites on the nitrocellulose were blocked by incubation for 30 minutes at room temperature with gentle agitation in 250 ml of 0.1% Tween 20 in phosphate buffered saline (PBS). Then the blot was washed three times with gentle agitation at room temperature for five minutes each in 250 ml volumes of 0.1% nonfat dry milk, 0.1% ovalbumin in PBS, and incubated with gentle agitation for three hours at room temperature in 5 ml of a 1/500 to 1/1000 dilution in the preceding buffer of rabbit antiserum against ricin A subunit or ricin B subunit (supplied by BABCO). After washing three times as described above, the blot was incubated for one hour at room temperature with 5 ml of a 1/2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase (this conjugate supplied by Zymed) and washed again three times as above.

In order to visualize the immobilized horseradish peroxidase immunoconjugate, the immunoconjugate-treated blot was soaked at room temperature without agitation for five minutes in 50 ml of 10 mM Na citrate, 10 mM EDTA, pH 5 and then for 15 minutes in 50 ml of freshly prepared 10 mM Na citrate, 10 mM EDTA and 1% dextran sulfate. In a glass container the blot was next agitated for 15 min in 50 ml citrate buffer (above) containing a 0.1 mg/ml tetramethylbenzidine (TMB added from a 2 mg/ml stock solution in ethanol; Miles Laboratories) then H_2O_2 was added to 0.0015%. When the pattern had reached the desired degree of contrast between specifically stained bands and background, the blot was soaked for 30-60 minutes in 50 ml of ice cold water and the blot was photographed.

E.9. Cytotoxicity Assay

Culture supernatants from Sf-9 cells were diluted 1:10 and 1:100 in culture medium and applied to RAT-2 cells (a fibroblast line transformed by Harvey murine sarcoma virus DNA; Clark, R. et al. (1985) Proc Natl. Acad. Sci., 82:5280). One series of 1:10 diluted samples were adjusted first to contain 50 mM lactose which is known to specifically block binding of native ricin toxin to the cell surface (Olsnes, S. et al. (1982) supra). These samples become the negative control. For positive controls, various concentrations of ricin toxin are prepared in Sf-9 cell culture medium. After 4 hours at 37°C, the samples were replaced with fresh medium and incubation was continued for 20 hours. The cell monolayers were then stained with crystal violet and visually analyzed for viability.

E.10. Assays for Binding Ricin B Chain Muteins

The ability of ricin B chain muteins according to the invention to bind to galactose is determined by measuring the binding of ricin B chain to galactose-containing resins or surfaces coated with galactose-containing molecules or membranes which contain galactose residues. Agarose-containing resins such as Sepharose, treated with dilute acid to expose galactose residues, Bio-Gel A resins, resins such as Sepharose or Bio-Gel A coupled to saccharides such as fetuin, are suitable for binding ricin with an intact B chain or ricin B chain alone. Ricin or ricin B chain in which the galactoside binding site or sites thereof has not been modified according to the invention when passed through these columns binds to the column material. If the galactoside-binding site has been altered to produce a ricin B mutein according to the invention, binding to galactoside will be measurably diminished or eliminated.

The ability of the ricin B mutein to bind galactose is further determined by equilibrium dialysis using 3H-labeled galactose. Fluorescent polarization techniques using methylumbelliferyl galactose may be used to measure the association of the galactose derivative with ricin B chain.

When ricin B muteins according to the invention are produced independently of ricin A chain, the ability of the ricin B mutein to interact with ricin A chain is determined by adding the ricin B mutein to a concentration of ricin A chain, deglycosylated ricin A chain, or recombinant ricin A chain that does not by itself inhibit protein synthesis in cells such as MCF-7 or HSB-2 cells. In order for the added ricin B chain to convert ricin A chain into a toxin, the galactose binding sites on ricin B chain must be capable of binding galactose-containing receptors on the surface of the target cell and the two chain must interact, either covalently through a disulfide bond or non-covalently. The ability of ricin B chain muteins according to the invention encoded by DNA containing alterations in the galactose binding regions as outlined above, to convert ricin A chain into a toxin is substantially decreased or absent.

The ability of ricin B chain muteins according to the invention to interact with ricin A chain is measured by a competition assay. Native ricin B chain, having intact galactose binding sites, interacts with ricin A chain and when the ricin A-ricin B complex is added to cells, protein synthesis is prevented. Ricin B chain muteins according to the invention made from DNA in which the galactose binding residues have been modified as outlined above is added to ricin A chain before or at the same time as native ricin B chain. The ricin B chain muteins according to the invention reduce the amount of measurable protein synthesis inhibition because the ricin B mutein displaces native ricin B chain in the complex. The extent of reduction in protein synthesis is proportional to the concentration of the competing mutant ricin B chain.

If the cysteine at position 4 in ricin B chain is capable of reacting with the cysteine in ricin A chain that forms the disulfide bridge between the two chains, gel electrophoresis in non-reducing polyacrylamide gels shows the presence of a 65,000 molecular weight species. Ricin A chain has a molecular weight of about 30,000 and ricin B chain has a molecular weight of about 33,000 (both native species). Since the native species are glycosylated, their apparent size will be larger than the recombinant molecules, if they are made in non-glycosylating prokaryotic cells.

Also within the scope of the invention are conjugates of ricin in which the B chain thereof is a mutein which has reduced binding to galactosides. Ricin is covalently bound to a binding moiety that can bind to a selected target cell or tissue and which can be internalized by such target cell or tissue. Such binding moieties may be selected from a vast number of substance that bind to specific cells or tissues and include lymphokines such as interleukin-1, 2 and 3 and interferon α' β and γ; cytokines such as tumor necrosis factor and colony stimulating factors such as, CSF-1, G-CSF and GM-CSF; hormones that bind to specific hormone receptors associated with specific tissues such as the reproductive hormones that bind to ovarian tissue, e.g., leutinizing hormone; cell growth factors such as transferrin and epidermal growth factor and antibodies that bind specifically to a desired target cell or which bind to an epitope that is expressed at high level on a target cell as compared to other cell or normal cells. Such antibodies may be polyclonal or monoclonal antibodies.

EXAMPLE I

A. Construction of New Baculovirus Transfer Vectors

A.1. Construction of pAcC1

pAcC1 is similar to pAc401 (described previously in Section C.5.) except that the recognition site for EcoRI endonuclease has been removed. To accomplish this, pAc401 was digested to completion with EcoRI and the ends were made blunt using Klenow fragment. After ligation and transformation, candidates were screened for the absence of an EcoRI site.

A.2. Construction of pAcC2

pAcC2 is similar to pAc436 (described previously in Section C.5.) except that the recognition site for EcoRI endonuclease has been removed. To accomplish this, pAc436 was digested to completion with EcoRI and the ends were made blunt using Klenow fragment. After ligation and transformation, candidates were screened for the absence of an EcoRI site.

A.3. Construction of pAcC3

pAcC3 differs from pAcC2 in that an NcoI restriction site has been introduced at the ATG translational start of the polyhedrin gene. To accomplish this the new transfer vector, pAcC2, was digested to completion with SmaI endonuclease. Following phenol extracton and ethanol precipitation, SmaI digested pAcC2 was dissolved in TE buffer (10 mM Tris-HCl pH 7.4; 1 mM EDTA). In a 50 µl volume of ExoIII buffer (50 mM Tris-HCl pH 8.0; 5 mM $MgCl_2$; 10 mM β-mercaptoethanol), 10 µg of SmaI digested pAcC2 was treated with 50 units of *E. coli* Exonuclease III (ExoIII) at 30°C for 5 minutes. The sample was phenol extracted and ethanol precipitated twice. Then 50 pmoles of a primer EK85, 5'-AACCTATAAACCATGGCGGCCCGG-3', was kinased with cold ATP in a 20 µl reaction volume (50 mM Tris-HCl pH 7.8; 10 mM $MgCl_2$; 10 mM β-mercaptoethanol). To 5 µg of ExoIII treated pAcC2 was added 10 pmoles of kinased EK85 in a final volume of 20 ml NET (100 mM NaCl; 10 mM Tris-HCl pH 7.5; 1 mM EDTA) buffer. To anneal the plasmid and primer, the reaction was heated to 65°C for 10 minutes, incubated at 37°C for 10 minutes and placed on ice. The extension reaction was performed by adding 20 µl 2 x Klenow buffer (40 mM Tris-HCl pH 7.5; 20 mM MgCl2; 2 mM β-mercaptoethanol) containing 1 µl 10 mM dNTPs, 1 µl 10 mMATP, 1 µl (about 2 units) Klenow fragment and 1 µl (about 1-2 units) T4 DNA ligase. The reaction was incubated at 16°C for about 4 hours and then transformed into MM294. Minilysates were screened by analyzing for the presence of an NcoI site. Miniprep DNA was then used to retransform and obtain the desired pure clone.

A.4 Construction of pAcC4 and pAcC5

pAcC4 and pAcC5 are derivatives of pAcC3 containing a polylinker sequence at the SmaI site. The polylinker contains recognition sites for restriction endonucleases SmaI, KpnI, PstI, BalII, XbaI (cleavable when DNA is unmethylated), EcoRI, BamHI and BclI. pAcC4 contains the sequence in one orientation while pAcC5 contains the polylinker in the opposite orientation (see Figure 4). To construct these vectors pAcC3 was digested to completion with XmaI endonuclease and ligated with two complementary self-annealed oligomers having the sequence:

```
5'-CCGGGTACCTGCAGATCTAGAATTCGGATCCTGATCA-3'
3'-    CATGGACGTCTAGATCTTAAGCCTAGGACTAGTGGCC-5'
```

After transformation of MM294, miniprep DNAs of transformants were analyzed for the presence of restriction sites in the polylinker sequence.

EXAMPLE II

Sensitivity of insect cells to ricin toxin

The insect host cell line, Sf-9, and ribosomes isolated from this cell line were tested for resistance to ricin toxin and ricin A chain, respectively.

The Sf-9 host cells were found to be unaffected by a 4 hour exposure to ricin D at concentrations of up to 10 µg/ml. After washing, the Sf-9 cells were incubated an additional 20 hours and remained unaffected. A derivative of the mouse 3T3 cell line called psi-2 (Mann, R. et al. (1983) Cell, 33:153), however, was almost completely inhibited/killed by a 4 hour exposure to ricin D at a concentration of 1 ng/ml. The ID50 (concentration at which 50% of the cells are inhibited/killed) for ricin on a sensitive cell line is typically 1-10 ng/ml.

The observed resistance of insect cells to ricin is apparently not due to the lack of toxin binding to the cell surface. In a sandwich-type binding assay using anti-ricin A sera for detection, a strong signal for ricin binding to the insect cells was observed. The cell surface moiety to which ricin A is apparently bound has not been characterized.

Ribosomes from Sf-9 cells appear to be resistant to ricin A chain. Sf-9 ribosomes were prepared according to Palmiter, R.D. (Biochem (1974) 13:3606) and tested in an in vitro translation assay (Cawley, D.B. et al. Biochem (1979) 12:2648) for sensitivity to added ricin A chain. At a concentration of recombinant ricin A of about 10 µg/ml, translation activity was depressed about 50%. Typically, 1-10 ng/ml of recombinant ricin A inhibits the rabbit reticulocyte translation system by 50%.

EXAMPLE III

Expression of Recombinant Ricin Toxin and Its Subunits

A. Construction of Recombinant Baculovirus Transfer Vectors Containing Full Length Ricin Toxin Sequences

The ricin sequence was obtained as a *Hind*III-*Hind*III fragment from a vector in which a *Hind*III site had been created at the ATG translational start at the beginning of the secretory signal peptide sequence as previously described. The *Hind*III site after the ricin gene can be derived from M13 or other suitable vectors carrying polylinker sequences. The *Hind*III fragment containing the ricin gene was made blunt ended with Klenow fragment and ligated to *Sma*I digested pAcC3. Recombinants containing the ricin sequence in the correct orientation for expression under polyhedrin promoter control were identified by restriction analysis. Two correct constructs were selected for transfection into baculovirus and were designated pBRT8 and pBRT15.

The sequence across the fusion junction and the expected amino acid sequence of the translation product are shown below.

```
polyhedrin 5' leader +1              preproricin sequence

    5'-AACCTATAAACC ATG GCG GCC CAG CTT ATG AAA CCG GGA-----------
                    Met Ala Ala Gln Leu Met Lys Pro Gly-----------
```

The native sequence in this construction is expected to be expressed with additional residues on its amino-terminus (in this case five) but these would be eliminated along with the signal peptide upon secretion.

B. Construction of Recombinant Baculovirus Transfer Vectors for Ricin B Subunit

1. As an Intracellular Product

The sequence encoding ricin B subunit was taken from pRTB601 on a *Hind*III-*Hind*III fragment. The fragment was treated with Klenow enzyme and all 4 dNTP's to blunt repair the sticky ends and was subsequently ligated into SmaI-digested pAcC3. Two recombinants containing the ricin B chain sequence in the correct orientation were identified and designated pBIB4 and pBIB24. The expected fusion sequence for expression is shown below.

```
polyhedrin 5' leader +1                      ricin B chain sequence
    5'-AACCTATAAACC ATG GCG GCC CAG CTT ATG GCT GAT--------------
                    Met Ala Ala Gln Leu Met Ala Asp
```

It was hoped that the six additional residues on the amino-terminus would not affect ricin B chain activity and that by keeping this short vector sequence at the beginning of the ricin B sequence, the efficiency of translational initiation would less likely be affected.

2. As a Secreted Product

The sequence encoding ricin B was taken from pRTB601 on a HindIII-HindIII fragment. After cloning this fragment into M13, site-specific mutagenesis using the primer, 5'-GTGCCAAGCTTTGCGCAGATGTTTGT-3' was carried out to introduce an FspI restriction site (underlined in primer) including the amino-terminal alanine codon (GCA) of the B subunit coding sequence. The B subunit coding sequence was then excised as an FspI-PstI fragment using the PstI site from the M13 vector at the 3' end of the B subunit insert.

The 97-mer encodes the CSF-1 secretory signal peptide (Kawasaki, E. et al., 1985, supra) except for four modifications at nucleotides 5, 10, 82 and 97 which were third position codon changes to create restriction recognition sites. Only the change at position 5 resulted in an amino acid coding change (Thr to Ala). The 97-mer having the sequence 5'-CATGGCCGCCCCGGGCGCCGCCGGGCGCTGCCCTCCCACGACATGG CTGGGCTCCCTGCTGTTGTTGGTCTGTCTCCTGGCCAGCAGGAGTWTCACG-3' and its complement when annealed have a 5' NcoI sticky end for insertion into

The transfer vector and a 3' blunt end for fusion to the FspI blunt end at the 5' end of the B subunit coding sequence. The transfer vector, pAcC5, was doubly digested with NcoI and PstI. A three fragment ligation resulted in a baculovirus transfer vector containing a CSF-1 secretory peptide fused to the ricin B subunit. This vector is designated pBSB1.

C. Construction of Recombinant Baculovirus Transfer Vectors for Ricin B Muteins

Native ricin B has asparagine at positions 46 and 255. Muteins were generated wherein glycine was substituted for asparagine at either one or both of these positions. Standard M13 cloning techniques were used to realize the 255 mutein, and the techniques described by Mandecki, W., Proc. Natl. Acad. Sci. USA 83, 7177 (1986) were used to produce the 46 mutein. The oligomer used to mutagenize asparagine at position 255 to glycine was GGTGACCCAGGTCAAATATGGTTACC, while the oligomer used to mutagenize the asparagine at position 46 to glycine was GTGGCCATGCAAGTCTAATACAGATGCCGGCCAGCTCTGGACTTTGAAA. In order to perform this mutagenesis it was previously necessary to introduce a unique restriction site near position 46 using standard mutagenesis techniques and the oligomer GCAAATCAGCTCTGGACTTTG.

1. Production of Transfer Vector pBG255

Briefly, RB was obtained from pRTB601 as a HindIII cassette, and using the oligomer shown above, asparagine at position 255 was converted to glycine in M13MP18. After mutagensis and confirmation of the mutagenized insert as described below, M13MP18 containing the mutagenized RTB DNA sequence was digested using HindIII, and the mutagenized sequence ligated into plasmid pPL231, thereby producing plasmid pPL231-RTB/AE82. The transfer vector, pBG255 was then constructed as shown in Figure 5 using the plasmid pBSB1, and pPL231-RTB/AE82.

The plasmid pBSB1 was treated with the following enzymes PstI, Klenow enzyme, BglII, and bacterial alkaline phosphatase. This treatment results in the excision of a ricin B encoding fragment and vector sequences, downstream of the fragment. The remaining large fragment, which exhibits ricin B sequences uptream of the BglII site, was ligated to a BglII-HgiAI fragment which contains sequences of ricin B wherein the asparagine amino acid at position 255 was site-specific mutagenized in M13 to Glycine. The BglII-HgiAI fragment was derived from the plasmid pPL231-RTB/AE82 by subjecting the plasmid to HgiAl, Klenow, and BglII treatment, and by gel purifying the BglII-

HgiAI fragment. Ligation of the large fragment obtained from pBSB1 to the BglII-HgiAI fragment yields the plasmid, pBG255 as shown in Figure 5.

A transfer vector, pBG46/255, containing a RB insert having asparagines at positions 46 and 255 mutated to glycine was constructed as shown in Figure 6. First, a RB construct was generated wherein asparagine at position 46 was mutated to glycine which consisted of inserting RB removed from pRTB601 as a HindIII cassette into pDG141. Next, the "oligomer overlay" technique of Mandecki, referred to above, was used to perform the mutagensis employing the oligomer also shown above. This procedure requires a restriction site in the vicinity of the sequence to be mutagenized .such that sequences which flank the sequence are complementary to the oligomer. Because there is a unique PvuII site near position 46 of RB, and because pDG141 does not have any PvuII restriction sites, it was possible to perform the overlay technique which produced plasmid pDG141 harboring the asparagine to glycine mutation. Next, pDG141 was digested with HindIII thereby removing the RB cassette containing glycine at position 46. The cassette was inserted into pPL231 thereby providing pPL231-RTB/AE98.

2. Production of Transfer Vector pBG46/255 and pBG46

pBG255 described above was restricted with BamHI, and treated with bacteria alkaline phosphatase. This treatment results in the excision of the middle portion of ricin B from the vector, while leaving associated with the large vector fragment ricin sequences on the upstream and downstream side of the removed fragment. Downstream of the fragment is the amino acid 255 which was mutagenized from asparagine to glycine. The large fragment resulting from BamHI digestion was ligated to a BamHI-BamHI fragment obtained from plasmid pPL231-RTB/AE98. This plasmid, which contains ricin B having had the asparagine amino acid at position 46 mutagenized to glycine, was ligated to the large fragment resulting from BamHI digestion of pBG255 thereby yielding the plasmid pBG46/255 having full length ricin B wherein the amino acids at positions 46 and 255 have been mutagenized from asparagine to glycine. Details of the construction of pBG46/255 are shown in Figure 6.

Transfer vector pBG46 was constructed using the same general materials and methods used to produce pBG46/255 with the exception that pBSB1 was substituted for pBG255.

It will be appreciated that details regarding some of the vectors described above are shown in European Publication No. 196,762, published October 8, 1986.

D. Construction of Recombinant Baculovirus Transfer Vectors for Ricin A Subunit

The sequence encoding ricin A subunit is contained on a *Hind*III/*Bam*HI restriction fragment as described earlier. This fragment was Klenow repaired and ligated into *Sma*I digested pAcC3 analogous to the construction in Section B.1.a. Transformants of *E. coli* MM294 were screened by restriction analysis of minilysates.

EXAMPLE IV

Expression of Ricin Toxin and its Subunits in Insect Cells

A. Expression of Full Length Toxin

pBRT8 and pBRT15 were each cotransfected into Sf9 cells with wild-type baculovirus DNA. Recombinant viruses were selected and plaqued a second time. The data discussed below are for second-round plaqued viruses. Each virus plaque was infected into two separate wells of cells. One was Sf-9 cells grown in standard medium containing 10% fetal calf serum. This infection was for virus propagation and to assay for secreted activity. The second was Sf-9 cells adapted to and grown in protein-free medium. This infection was to produce samples for SDS-PAGE and Western blot analyses on the medium. After 4-5 days, the culture supernatants were collected and assayed. Cytotoxicity assays were run on the first set of supernatants. Aliquots were diluted 1:10 and 1:100 into medium and placed onto RAT-2 cells (this is a tk^- variant of the rat cell line, RAT-1) for 4 hours, replaced with fresh medium, and examined 20 hours later. The results are shown in Figure 7. The infected cell supernatants from the BRT15 plaques, 6-1 and 6-2, show definite cytoxicity at both 1:10 and 1:100 dilutions that can be blocked by the addition of 50 mM lactose. Lactose is able to block specific binding of native ricin. Support for this being ricin-like activity comes from the Western blot analysis of the second set of infected supernatants. As shown in Figure 8, immunoreactive proteins are clearly present in the BRT15, 6-1 and 6-2 samples (lanes 7 and 8), and are much less, if at all, present in the other samples. Lane 5 is the cell supernatant from Sf9 cells infected with wild-type baculovirus. Compared with native ricin standards (lane 1), each of the two supernatants (lanes 7 and 8) contain a product that migrates close to the position of intact ricin that is detected by both the anti-RTA and anti-RTB sera. This may correspond to secreted product that is not processed further into the A and B subunits. It represents perhaps 60-70% of the total detected products. The anti-RTA sera also detects two products which migrate approximately with native ricin A1 and A2 chains and likely are analogous to those forms of ricin A chain. The anti-RTB sera detects additionally a product that migrates approximately with native ricin B chain. These smaller products collectively comprise the remainder of the 30-40% of the detected material. These results demonstrate that the baculovirus expression vector system can express, secrete and process into its subunits, active ricin toxin.

B. Expression of Ricin B

1. pBIB4 and pBIB24 DNAs were each cotransfected into Sf9 cells with wild type baculovirus DNA.

The initial transfected cells and cells infected with the subsequently plaque-purified viruses were assayed for ricin B chain expression to aid in selecting recombinant viruses. The data discussed below was taken from experiments in which either once or twice plaque-purified virus was used. Infected cells were harvested 4-6 days after infection, when the cultures showed a high percentage of late-infected cells.

By Western blot analysis of several pBIB4 and pBIB24 first-round plaqued viruses, it was clear that a ricin B chain protein was expressed in most, but not all, plaque-pool infections. "False" recombinant viruses may be attributed to a natural loss of the polyhedrin phenotype as there is no selection for it _in vitro_. The ricin B chain product expressed has a molecular weight of 29 kD (see Figure 9, lane 5), consistent with that expected for a non-secreted, non-glycosylated product. In other analyses (data not shown) of second-round plaqued viruses, the same product was noted by Western blot but could not be visualized in Coomassie stained gels. From a comparison of signal strengths, it was estimated that the ricin B chain is produced at about 2 microgram/ml. Cells were disrupted by sonication and upon low speed centrifugation this product distributed approximately equally between the supernatant and pellet fractions.

2. The transfer vector, pBSB1, containing the CSF-1 secretory peptide fused to ricin B was transfected with baculovirus DNA into Sf-9 cells and recombinant virus was selected. Ricin B was detected by Western blot analysis as described by Towbin et al., (1979) _Proc. Natl. Acad. Sci. USA_, _76_:4350. Rabbit anti-sera to ricin B-chain was utilized. It was observed that ricin B chain is expressed as two cell associated proteins, one having a molecular weight of about 32,000. The 32,000 and 36,000 species accumulated to significant levels in the range of about 10 μg/ml and 2 mg/ml, respectively. Of this, approximately 10 nanograms/μl was active B chain. Additionally, it was observed that ricin B chain was also expressed as a secreted protein having a molecular weight of about 36,000. This molecule accumulates to significant level in the range of about 2 mg/ml. Of this, approximately 10 nanograms/per ml was active B chain. Additionally, a third form of B chain was detected in the culture supernatant. It has an apparent molecular weight of 32,000 and was present in the culture supernatant at about 10 nanogram/per ml or greater levels.

C. Expression of Ricin B Muteins

The materials and methods used to express ricin B muteins was essentially similar to that described for the expression of ricin B above, with the exception that generally higher levels of the mutein was observed in culture supernatant and were in the range of at least 20 nanograms/per ml.

D. Expression of Ricin A

After transfection of the recombinant transfer vector and baculovirus DNA into Sf-9 cells, recombinant viruses were selected. There is no evidence of ricin A production by either bioactivity assay or Western blot analysis.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the scope of this invention.

EXAMPLE V

Purification of Ricin B Muteins

A. Purification of Ricin B Chain Double Mutant

The ricin B chain double mutant described in Example VI.C above was purified from Sf9 culture medium. Approximately 5.5 liters of culture medium in which Sf9 containing the baculovirus expression vect~~h~~or pBG46/255 cell were grown was adjusted to a pH of about 7.2 and applied to a Zetaprep-QAE cartridge, obtainable from AMF Molecular Separations, Catalog No. 1600 1501 00QA. Prior to applying the culture media to the cartridge, the cartridge was activated by first passing 0.1 molar tribasic sodium phosphate, pH 9, through the cartridge, and then 0.1 molar sodium acetate, pH 3. Next the cartridge was washed with 0.1 molar sodium phosphate, pH 7.2 until the pH fluid eminating from the cartridge was 7.2, at which time it was further washed with 10 mM sodium phosphate, pH 7.2, with 40 mM NaCl until the conductivity of buffer entering and leaving the cartridge was about 4 millisiemens/cm.

The culture medium was passed through the cartridge and the cartridge then washed with 10 mM sodium phosphate, pH 7.2, containing 40 mM NaCl until such time that the absorbance reading at 280 nm was 0. Next, the protein which bound to the cartridge was eluted in a single step gradient with 10 mM sodium phosphate, pH 7.2, containing 1 M NaCl. Those fractions containing the mutein were identified by immunoblots using affinity purified-antibody raised against naturally occurring ricin B chain. It was calculated that the concentration of the ricin B chain mutein was about 240 nanograms/per ml.

EXAMPLE VI

A. Galactose Binding Activity of Ricin B Muteins

In order to ascertain whether the substitution of glycine for asparagine at positions 46 and/or 255 of ricin B reduced galactose binding activity, an assay was employed wherein binding of the muteins was compared to binding of native ricin B chain. The assay consisted of determining if these molecules bound to asialofetuin which was absorbed to the bottom of a 96-well plastic tissue culture plate. Asialofetuin was obtained from Sigma Corporation, and was dissolved at 1 mg/per ml in phosphate buffered saline containing 0.5% bovine serum albumin, 0.05% Tween-20, and the preservative thimersal at 0.01%. More specifically, the procedure involved diluting the asialofetuin solution to a concentration of 5 μg per ml with 0.05 molar sodium carbonate, pH 9.6, and 100 microliters of this solution was added to wells in the 96 well plate. The solution was allowed to incubate overnight at 4°C so as to provide maximum time for asialofetuin to adhere to the tissue culture plates. Next, the plates were washed with phosphate buffered saline to remove unattached asialofetuin, and then areas on the culture tissue wells which did not bind asialofetuin were blocked with bovine serum albumin in phosphate buffered saline, Tween-20 and thimerisal for one hour at room temperature. The plates were washed a second time with phosphate buffered saline containing

Tween-20, and subsequently 100 microliters of either native ricin B, or the mutein was added to each well. The samples were diluted into phosphate buffered saline containing 0.5% bovine serum albumin before addition to the wells, and where necessary, lactose was also present. The samples were incubated for two hours with moderate shaking at 21°C and the plates were washed with phosphate buffered saline containing Tween-20 to remove unbound reactants. Subsequently, 100 microliters of polyclonal rabbit anti-ricin B chain antibody, conjugated to horseradish peroxidase, previously diluted in the range of 1:1000 to 1:5000 was added to each of the wells and incubated for two hours with shaking at 21°C. The wells were further washed with PBS containing Tween-20 and 100 microliters of a solution containing horseradish peroxidase substrate, ortho-phenylenediamine, was added and allowed to react for 15 minutes prior to stopping the reaction with 100 microliters of two normal sulfuric acid. The substrate was made by dissolving a 30 mg tablet (Sigma lot. 36F-8815 No. P-8412) in 50 ml of sodium phosphate-citrate buffer/urea peroxide. The amount of antibody bound to either native ricin or the mutein was ascertained by measuring the absorbance of the solution at 490 and 405 nm.

Figure 10 shows that native ricin B chain at three different concentrations, 10, 50, and 100 ng per ml, binds efficaciously to asialofetuin. It further shows the expected result: as lactose concentration is increased the amount of native ricin B chain displaced from asialofetuin is reduced considerably and this is dependent on the amount of native ricin B chain initially present in the reaction solution. In contrast, Figure 11 shows that ricin B chain mutein having the asparagine at position 255 mutated to glycine exhibits markedly reduced binding to asialofetuin, and that considerably less lactose is required to prevent the binding of the single amino acid mutein. It was further observed, that the ricin B chain double mutein having amino acids at positions 46 and 255 changed from aspargine to glycine exhibits no binding, or at least binding which is so low as not to be detectable by the assay utilized.

This invention has been described in detail including the preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the scope of this invention.

Deposits

The materials listed below were deposited with the American Type Culture Collection, Rockville, MD, USA (ATCC). The deposits were made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty). Maintenance of a viable culture is assured for 30 years from date of deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Applicants and ATCC which assures unrestricted availability upon issuance of the pertinent U.S. patent. Availability of the deposited strains is not to be construed as a license to practice the invention in contravention rights granted under the authority of any government in accordance with its patent laws.

Recombinant Transfer Vector	CMCC#	ATCC#	Deposit Date
pBRT8	3115	67434	6/12/87
pBRT15	3116	67435	6/12/87
pBIB24	3114	67433	6/12/87
pBSB1	67562		11/21/87
pDG141	39588		1/24/84
pBG46			
pBG255			
pBG46/255			

WHAT IS CLAIMED IS:

1. A recombinant baculovirus transfer vector capable of introducing a ricin toxin gene or portion thereof into a baculovirus genome said transfer vector comprising a portion of the baculovirus genome, a bacterial replication origin and a DNA sequence encoding a ricin toxin, subunit thereof or an amino acid sequence substantially equivalent to that of ricin toxin under the transcriptional control of a baculovirus promoter.

2. A recombinant baculovirus transfer vector according to claim 1 wherein said DNA sequence encoding a ricxin toxin or a subunit thereof is selected from the group of plasmids consisting of pRT17, pRA123 or pRTB601.

3. A recombinant baculovirus transfer vector according to claim 1 wherein said baculovirus promoter is the polyhedrin gene promoter.

4. A recombinant baculovirus transfer vector according to claim 3 wherein said DNA sequence encoding a ricin toxin or a subunit thereof is selected from the group of plasmids consisting of pRT17, pRA123 or pRTB601.

5. A recombinant baculovirus transfer vector according to claim 3 wherein a NcoI restriction endonuclease recognition site has been introduced at the ATG translational initiation codon of the polyhedrin gene.

6. A recombinant baculovirus transfer vector according to claim 3 wherein said ricin toxin gene or portion thereof is in proper translational reading frame with a heterologous secretory signal peptide.

7. pBRT8.

8. pBRT15.

9. pBIB24.

10. pBSB1.

11. A recombinant baculovirus transfer vector according to claim 1 wherein said subunit of ricin toxin is a mutein of ricin B comprising an alteration in at least one amino acid residue forming a part of at least one of the ricin B binding sites for galactosides wherein said amino acid forming at least a part of one galactoside binding site of ricin B comprises those amino acids that are within about 5 Angstroms of the galactoside in the 2.8 Angstrom resolution crystal structure of ricin, and are selected from the group consisting of Asp22, Arg24, Asp25, Gly26, Gln35, Trp37, Lys40, Asp44, Asn46, Asp234, Arg236, Ala237, Ile246, Tyr148, His251, Asn255 and Gln256.

12. A recombinant baculovirus transfer vector according to claim 11 wherein said amino acid alteration comprises deletion or substitution of at least one amino acid of the galactoside binding site that participates in hydrogen bonding to galactoside with a substituting amino acid that does not participate in hydrogen bonding to galactoside, wherein said at least one amino acid of the galactoside binding site participating in hydrogen bonding to galactoside is an amino acid selected from the group consisting of Arg24, Asp25, Gly26, Gln35, Lys40, Asp44, Asn46, Arg236, Ala237, Ile246, His251, Asn255 and Gln256, and said substituting amino acid that does not participate in hydrogen bonding of galactoside is selected from the group consisting of Gly and Ala.

13. A recombinant baculovirus transfer vector according to claim 11 wherein said amino acid alteration comprises deletion of an amino acid of the galactoside binding site that stabilizes an amino

acid that participates in hydrogen bonding to galactoside or the substitution of an amino acid of the galactoside binding site that stabilizes an amino acid that participates in hydrogen bonding to galactoside, with an amino acid that does not stabilize an amino acid that participates in hydrogen bonding to galactoside wherein said stabilizing amino acid is selected from the group consisting of Asp22 and Asp234, and said amino acid that substitutes for a stabilizing amino acid is selected from the group consisting of Gly, Ala and Ser.

14. A recombinant baculovirus transfer vector having a mutein of ricin B comprising at least one of Asp22, Arg24, Asp25, Gly26, Gln35, Trp37, Lys40, Asp44, Asp234, Arg236, Ala237, Ile246, Tyr248, His251, Asn255 and Gln256 substituted by an amino acid selected from the group consisting of Gly, Ala, Ser, Lys or Arg when the substituted amino acid is Asp or Gln, and Gln when the substituted amino acid is lys or Arg, said mutein having a lower affinity for galactoside than the corresponding unsubstituted form of ricin B.

15. A recombinant baculovirus transfer vector having a mutein of ricin B comprising at least one of Asp22, Arg24, Asp25, Gly26, Gln35, Trp37, Lys40, Asp44, Asn46, Asp234, Arg236, Ala237, Ile246, Tyr248, His251, Asn255 and Gln256 deleted, said mutein having a lower affinity for galactoside than the corresponding unsubstituted form of ricin B.

16. A recombinant baculovirus transfer vector having a mutein of ricin B comprising at least one of Asp22, Arg24, Asp25, Gly26, Gln35, Trp37, Lys40, Asp44, Asn46, Asp234, Arg236, Ala237, Ile246, Tyr248, His251, Asn255 and Gln256 substituted by an amino acid selected from the group consisting of Gly, Ala, Ser, Lys or Arg when the substituted amino acid is Asp or Gln, and Asp or Glu when the substituted amino acid is Lys or Arg, and at least one of the remaining unsubstituted amino acids deleted, said mutein having a lower affinity for galactoside than the corresponding undeleted unsubstituted form of ricin B.

17. A recombinant baculovirus transfer vector having a mutein of ricin B, said ricin B comprising an amino and a carboxyl galactoside binding site, said mutein comprising the substitution of two amino acids of at least one of said galactoside binding sites, said two amino acids of the amino binding site selected from the group consisting of residues 22, 25, 35, 37 and 46, said two amino acids of the carboxyl binding site selected from the group consisting of residues 234, 248, 251 and 255 wherein said substitution is either Gly or Ala.

18. A recombinant baculovirus transfer vector having a mutein of ricin B, said ricin B comprising an amino and a carboxyl galactoside binding site, said mutein comprising the substitution of one amino acid of at least one of said galactoside binding sites, said one amino acid of the amino binding site selected from the group consisting of residues 22, 25, 26, and 46, said amino acid of the carboxyl binding site selected from the group consisting of residues 234 and 255, wherein said substitution is either Leu or Ile.

19. A recombinant baculovirus transfer vector having a mutein of ricin B, said ricin B comprising an amino and a carboxyl galactoside binding site, said mutein comprising the substitution of two amino acids of at least one of said galactoside binding sites, said two amino acids of the amino binding site selected from the group consisting of residues 22, 25, 26, 34 and 46, said amino acid of the carboxyl binding site selected from the group consisting of 234, 248, 251 and 255 wherein when the substitution at amino acids selected from the group consisting of 22, 25, 26, 34 and 46 is either Leu or Ile, the other substitution of the selected amino acids of the amino binding site is Gly or Ala and when the substitution at amino acids selected from the group consisting of 234 and 255 is either Leu or Ile, the other substitution of the selected amino acids of the carboxyl binding site consisting of 234, 248, 251 and 255 is Gly ofr Ala.

20. A recombinant baculovirus transfer vector having a mutein of ricin toxin or the precursor thereof wherein the ricin B chain thereof comprises the ricin B mutein of claims 11, 12, 13, 14, 15, 16, 17, 18 and 19.

21. A recombinant baculovirus transfer vector having a DNA sequence encoding the mutein of ricin B of claims 11, 12, 13, 14, 15, 16, 17, 18, or 19.

22. A recombinant baculovirus transfer vector having a DNA sequence encoding the mutein of ricin toxin or the precursor thereof of claim 20.

23. A recombinant baculovirus expression vector for the production of biologically active ricin toxins, ricin toxin subunits or proteins having an amino acid sequence substantially equivalent to that of ricin toxin, said expression vector comprising a recombinant baculovirus genome and a DNA sequence substantially equivalent to that of ricin toxin under the transcriptional control of a baculovirus promoter, wherein said DNA sequence encoding said ricin toxin or subunit thereof is selected from the group of plasmids consisting of pRT17, pRA123 or pRTB601, and said baculovirus promoter is the polyhedrin gene promoter.

24. A recombinant baculovirus expression vector having a mutein of ricin B comprising at least one of Asp22, Arg24, Asp25, Gly26, Gln35, Trp37, Lys40, Asp44, Asn46, Asp234, Arg236, Ala237, Ile246, Tyr248, His251, Asn255 and Gln256 substituted by an amino acid selected from the group consisting of Gly, Ala, Ser, Lys or Arg when the substituted amino acid is Asp or Gln, and Asp or Gln when the substituted amino acid is Lys or Arg, said mutein having a lower affinity for galactoside than the corresponding unsubstituted form of ricin B.

25. A recombinant baculovirus expression vector having a mutein of ricin B comprising at least one of Asp22, Arg24, Asp25, Gly26, Gln35, Trp37, Lys40, Asp44, Asn46, Asp234, Arg236, Ala237, Ile246, Tyr248, His251, Asn255 and Gln256 deleted, said mutein having a lower affinity for galactoside than the corresponding unsubstituted form of ricin B.

26. A recombinant baculovirus expression vector having a mutein of ricin B comprising at least one of Asp22, Arg24, Asp25, Gly26, Gln35, Trp37, Lys40, Asp44, Asn46, Asp234, Arg236, Ala237, Ile246, Tyr248, His251, Asn255 and Gln256 substituted by an amino acid selected from the group consisting of Gly, Ala, Ser, Lys or Arg when the substituted amino acid is Asp or Gln, and Asp or Glu when the substituted amino acid is Lys or Arg, and at least one of the remainaing unsubstituted amino acids deleted, said mutein having a lower affinity for galactoside than the corresponding undeleted unsubstituted form of ricin B.

27. A recombinant baculovirus expression vector having a mutein of ricin B, said ricin B comprising an amino and a carboxyl galactoside binding site, said mutein comprising the substitution of two amino acids of at least one of said galactose binding sites, said two amino acids of the amino binding site selected from the group consisting of residues 22, 25, 35, 37 and 46, said two amino acids of the carboxyl binding site selected from the group consisting of residues 234, 248, 251 and 255 wherein said substitution is either Gly or Ala.

28. A recombinant baculovirus expression vector having a mutein of ricin B, said ricin B comprising an amino and a carboxyl galactoside binding site, said mutein comprising the substitution of one amino acid of at least one of said galactoside binding sites, said one amino acid of the amino binding site selected from the group consisting of residues 22, 25, 26 and 46, said amino acid of the carboxyl binding site selected from the group consisting of 234 and 255, wherein said substitution is either Leu or Ile.

29. A recombinant baculovirus expression having a mutein of ricin B, said ricin B comprising an amino and a carboxyl galactoside binding site, said mutein comprising the substitution of two amino acids of at least one of said galactoside binding sites, said two amino acids of the amino binding site selected from the group consisting of residues 22, 25, 26, 34 and 46, said amino acid of the carboxyl binding site selected from the group consisting of 234, 248, 251 and 255, wherein when the substitution at amino acids selected from the group consisting of 22, 25, 26 and 46 is either Leu or Ile, the other substitution of the selected amino acids of the amino binding site is Gly or Ala and when the substitution at amino acids selected from the group consisting of 234 and 255 is either Leu or Ile, the other substitution of the selected amino acids of the carboxyl binding site consisting of 234, 248, 251 and 255 is Gly or Ala.

30. pBG46.

31. pBG255.

32. pBG46/255.

33. Proteins encoded by said baculovirus transfer vectors of claim 1.

34. Proteins encoded by said baculovirus transfer vectors selected from the group consisting of pBG46, pBG255 and pBG46/255.

35. Proteins encoded by the transfer vector pBG46/255.

36. Proteins produced by said baculovirus expression vector of claim 23.

37. Proteins produced by a baculovirus expression vectors having the ricin B mutein DNA sequences of plasmids selected from the group consisting of pBG46, pBG255 and pBG46/255.

38. Proteins produced by a baculovirus expression vector having the ricin B mutein DNA sequence of plasmid pBG46/255.

39. A method for producing a biologically active ricin toxin, ricin toxin subunits, or muteins or proteins thereof, having amino acid sequences substantially equivalent to that of ricin toxin, said subunits, muteins, or proteins comprising infecting a susceptible host insect cell with a recombinant baculovirus expression vector wherein said expression vector is a recombinant baculovirus genome comprising at least one DNA sequence encoding said ricin toxin, subunits, muteins or proteins thereof under the transcriptional control of a baculovirus promoter, growing said infected insect cells under suitable conditions and recovering said polypeptide from the culture medium.

40. The method of claim 39 wherein the recombinant baculovirus expression vector containing a gene encoding ricin toxin or one of its subunits is produced by recombination with a transfer vector selected from the group consisting of pBRT8, pBRT15, pBIB24, pBSB1, pBG46, pBG255, and pBG46/255.

FIG. 1

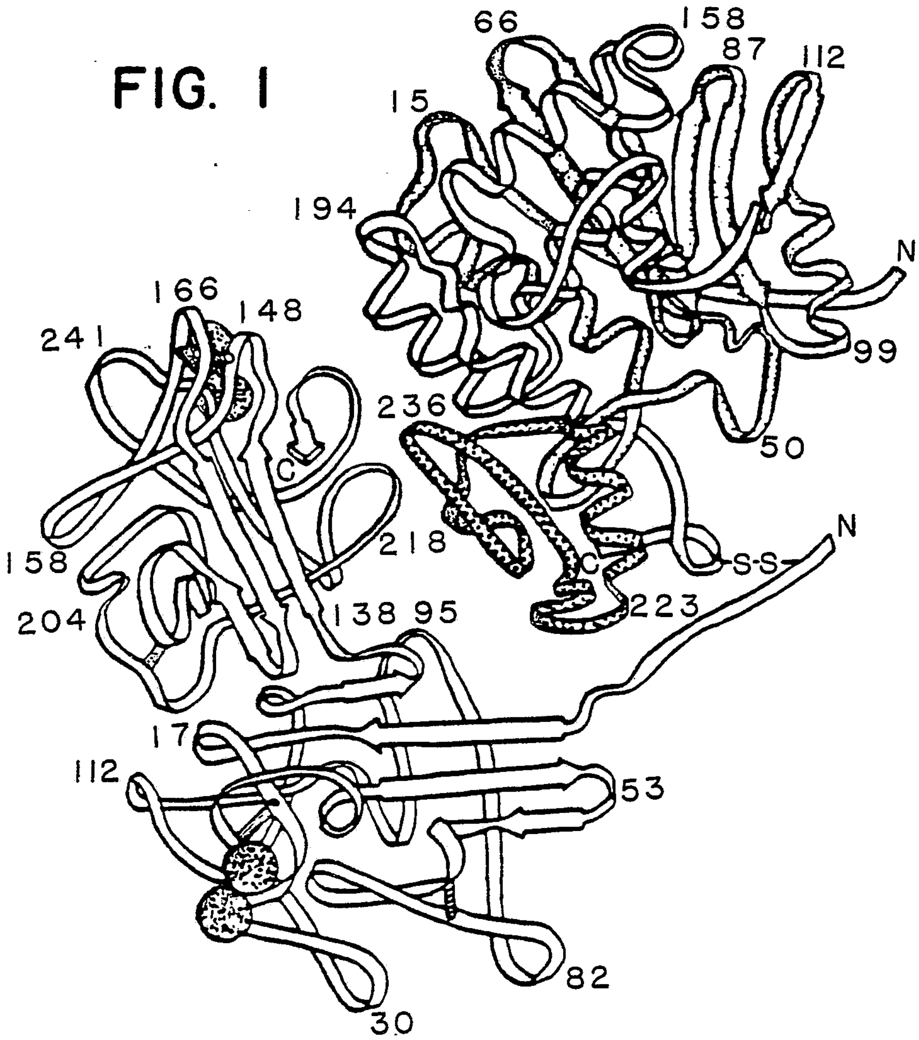

N
C
C
S-S
N

FIG. 2A

```
                                    Sal 1
                                    GTCGAC
5                         GGTCTATGTGTCGACGTTAGGG  oligonucleotide
CCCATAGTGCGTATCGTAGGTCGAAATGGTCTATGTGTTGATGTTAGGGATGGAAGATTC
---------+---------+---------+---------+---------+---------+ 1020
GGGTATCACGCATAGCATCCAGCTTTACCAGATACACAACTACAATCCCTACCTTCTAAG
3'                                  CAGCTG
P  I  V  R  I  V  G  R  N  G  L  C  V  D  V  R  D  G  R  F  -
  10            15             20             25

                                                      Pvu 2
                                                      CAGCTG
5'                                                GCAAATCAGCTG
CACAACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAGCTC
---------+---------+---------+---------+---------+---------+ 1080
GTGTTGCCTTTGCGTTATGTCAACACCGGTACGTTCAGATTATGTCTACGTTTAGTCGAG
3'                                                    GTCGTC
H  N  G  N  A  I  G  L  W  P  C  K  S  N  T  D  A  N  G  L  -
  30            35             40             45

TGGACTTTG oligonucleotide
TGGACTTTGAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTACGGG
---------+---------+---------+---------+---------+---------+ 1140
ACCTGAAACTTTTCTCTGTTATGATAAGCTAGATTACCTTTCACAAATTGATGAATGCCC
W  T  L  K  R  D  N  T  I  R  S  N  G  K  C  L  T  T  Y  G  -
  50            55             60             65
```

SUBSTITUTE SHEET

FIG. 2B

```
                                     Xba 1
                                    (TCTAGA)
                             GGGTTGGTTCTAGATGTGAGGG
     GGAACCATTTTAAATTTGTATAGTGGGTTGGTGTTAGATGTGAGGGCATCGGATCCGAGC
1600 +---------+---------+---------+---------+---------+--------- 1659
     CCTTGGTAAAATTTAAACATATCACCCAACCACAATGTACACTCCCGTAGCCTAGGCTCG
                                     AGATCA
     G  T  I  L  N  L  Y  S  G  L  V  L  D  V  R  A  S  D  P  S   -
                                        234

                                                           CCA
     CTTAAACAAATCATTCTTTACCCTCTCCATGGTGACCCAAACCAAATATGGTTACCATTA
1660 +---------+---------+---------+---------+---------+--------- 1719
     GAATTTGTTTAGTAAGAAATGGGAGAGGTACCACTGGGTTTGGTTTATACCAATGGTAAT
     L  K  G  I  I  L  Y  P  L  H  G  D  P  N  G  I  W  L  P  L   -
                               251         256
               Bac 2
              (CCGCGG)
     TTTTGATAGACCGCGGACTCTCTTGCAG
     TTTTGATAGACAGATTACTCTCTTGCAGTGTGTATGTCCTGCCATGAAAATAGATGGCTT
1720 +---------+---------+---------+---------+---------+--------- 1779
     AAAACTATCTGTCTAATGAGAGAACGTCACACATACAGGACGGTACTTTTATCTACCGAA
     F  *  *  T  D  Y  B  L  A  V  C  M  B  C  H  E  N  R  W  L   -
```

FIG. 3-1

```
   1 XAACCGGGAGGAAATACTATTGTAATATGGATGTATGCAGTGGCAACATGGCTTTGTTTT
        ProGlyGlyAsnThrIleValIleTrpMetTyrAlaValAlaThrTrpLeuCysPhe
      <----------------------------------------------------------

  61 GGATCCACCTCAGGGTGGTCTTTCACATTAGAGGATAACAACATATTCCCCAAACAATAC
     GlySerThrSerGlyTrpSerPheThrLeuGluAspAsnAsnIlePheProLysGlnTyr
     ----------------------- leader -----›||-----›A-chain NH2

 121 CCAATTATAAACTTTACCACAGCGGGTGCCACTGTGCAAAGCTACACAAACTTTATCAGA
     ProIleIleAsnPheThrThrAlaGlyAlaThrValGlnSerTyrThrAsnPheIleArg
     end (native)

 181 GCTGTTCGCGGTCGTTTAACAACTGGAGCTGATGTGAGACATGAAATACCAGTGTTGCCA
     AlaValArgGlyArgLeuThrThrGlyAlaAspValArgHisGluIleProValLeuPro

 241 AACAGAGTTGGTTTGCCTATAAACCAACGGTTTATTTTAGTTGAACTCTCAAATCATGCA
     AsnArgValGlyLeuProIleAsnGlnArgPheIleLeuValGluLeuSerAsnHisAla

 301 GAGCTTTCTGTTACATTAGCGCTGGATGTCACCAATGCATATGTGGTAGGCTACCGTGCT
     GluLeuSerValThrLeuAlaLeuAspValThrAsnAlaTyrValValGlyTyrArgAla

 361 GGAAATAGCGCATATTTCTTTCATCCTGACAATCAGGAAGATGCAGAAGCAATCACTCAT
     GlyAsnSerAlaTyrPhePheHisProAspAsnGlnGluAspAlaGluAlaIleThrHis

                        |ClaI|
 421 CTTTTCACTGATGTTCAAAATCGATATACATTCGCCTTTGGTGGTAATTATGATAGACTT
     LeuPheThrAspValGlnAsnArgTyrThrPheAlaPheGlyGlyAsnTyrAspArgLeu

 481 GAACAACTTGCTGGTAATCTGAGAGAAAATATCGAGTTGGGAAATGGTCCACTAGAGGAG
     GluGlnLeuAlaGlyAsnLeuArgGluAsnIleGluLeuGlyAsnGlyProLeuGluGlu

 541 GCTATCTCAGCGCTTTATTATTACAGTACTGGTGGCACTCAGCTTCCAACTCTGGCTCGT
     AlaIleSerAlaLeuTyrTyrTyrSerThrGlyGlyThrGlnLeuProThrLeuAlaArg

 601 TCCTTTATAATTTGCATCCAAATGATTTCAGAAGCAGCAAGATTCCAATATATTGAGGGA
     SerPheIleIleCysIleGlnMetIleSerGluAlaAlaArgPheGlnTyrIleGluGly

 661 GAAATGCGCACGAGAATTAGGTACAACCGGAGATCTGCACCAGATCCTAGCGTAATTACA
     GluMetArgThrArgIleArgTyrAsnArgArgSerAlaProAspProSerValIleThr

 721 CTTGAGAATAGTTGGGGGAGACTTTCCACTGCAATTCAAGAGTCTAACCAAGGAGCCTTT
     LeuGluAsnSerTrpGlyArgLeuSerThrAlaIleGlnGluSerAsnGlnGlyAlaPhe

 781 GCTAGTCCAATTCAACTGCAAAGACGTAATGGTTCCAAATTCAGTGTGTACGATGTGAGT
     AlaSerProIleGlnLeuGlnArgArgAsnGlySerLysPheSerValTyrAspValSer

 841 ATATTAATCCCTATCATAGCTCTCATGGTGTATAGATGCGCACCTCCACCATCGTCACAG
     IleLeuIleProIleIleAlaLeuMetValTyrArgCysAlaProProProSerSerGln
                                                     A-chain ----
         ctttgcttataaggagggtggtacc (4)
 901 TTTTCTTTGCTTATAAGGCCAGTGGTACCAAATTTTAATGCTGATGTTTGTATGGATCCT
     PheSerLeuLeuIleArgProValValProAsnPheAsnAlaAspValCysMetAspPro
     -›|                                    |-----› B-chain

 961 GAGCCCATAGTGCGTATCGTAGGTCGAAATGGTCTATGTGTTGATGTTAGGGATGGAAGA
     GluProIleValArgIleValGlyArgAsnGlyLeuCysValAspValArgAspGlyArg

1021 TTCCACAACGGAAACGCAATACAGTTGTGGCCATGCAAGTCTAATACAGATGCAAATCAG
     PheHisAsnGlyAsnAlaIleGlnLeuTrpProCysLysSerAsnThrAspAlaAsnGln
```

FIG. 3-2

```
1081 CTCTGGACTTTGAAAAGAGACAATACTATTCGATCTAATGGAAAGTGTTTAACTACTTAC
     LeuTrpThrLeuLysArgAspAsnThrIleArgSerAsnGlyLysCysLeuThrThrTyr

1141 GGGTACAGTCCGGGAGTCTATGTGATGATCTATGATTGCAATACTGCTGCAACTGATGCC
     GlyTyrSerProGlyValTyrValMetIleTyrAspCysAsnThrAlaAlaThrAspAla

1201 ACCCGCTGGCAAATATGGGATAATGGAACCATCATAAATCCCAGATCTAGTCTAGTTTTA
     ThrArgTrpGlnIleTrpAspAsnGlyThrIleIleAsnProArgSerSerLeuValLeu

1261 GCAGCGACATCAGGGAATAGTGGTACCACACTTACAGTGCAAACCAACATTTATGCCGTT
     AlaAlaThrSerGlyAsnSerGlyThrThrLeuThrValGlnThrAsnIleTyrAlaVal

1321 AGTCAAGGTTGGCTTCCTACTAATAATACACAACCTTTTGTGACAACCATTGTTGGGCTA
     SerGlnGlyTrpLeuProThrAsnAsnThrGlnProPheValThrThrIleValGlyLeu

1381 TATGGTCTGTGCTTGCAAGCAAATAGTGGACAAGTATGGATAGAGGACTGTAGCAGTGAA
     TyrGlyLeuCysLeuGlnAlaAsnSerGlyGlnValTrpIleGluAspCysSerSerGlu

1441 AAGGCTGAACAACAGTGGGCTCTTTATGCAGATGGTTCAATACGTCCTCAGCAAAACCGA
     LysAlaGluGlnGlnTrpAlaLeuTyrAlaAspGlySerIleArgProGlnGlnAsnArg

1501 GATAATTGCCTTACAAGTGATTCTAATATACGGGAAACAGTTGTCAAGATCCTCTCTTGT
     AspAsnCysLeuThrSerAspSerAsnIleArgGluThrValValLysIleLeuSerCys

1561 GGCCCTGCATCCTCTGGCCAACGATGGATGTTCAAGAATGATGGAACCATTTTAAATTTG
     GlyProAlaSerSerGlyGlnArgTrpMetPheLysAsnAspGlyThrIleLeuAsnLeu

1621 TATAGTGGGTTGGTGTTAGATGTGAGGGCATCGGATCCGAGCCTTAAACAAATCATTCTT
     TyrSerGlyLeuValLeuAspValArgAlaSerAspProSerLeuLysGlnIleIleLeu

1681 TACCCTCTCCATGGTGACCCAAACCAAATATGGTTACCATTATTTTGATAGACAGATTAC
     TyrProLeuHisGlyAspProAsnGlnIleTrpLeuProLeuPhe......
                                   B-Chain -----›|

1741 TCTCTTGCAGTGTGTATGTCCTGCCATGAAAATAGATGGCTTAAATAAAAAGGACATTGT

1801 AAATTTTGTAACTGAAAGGACAGCAAGTTATTGCAGTCCAGTATCTAATAAGAGCACAAC

1861 TATTGTCTTGTGCATCTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

1921 AAA
```

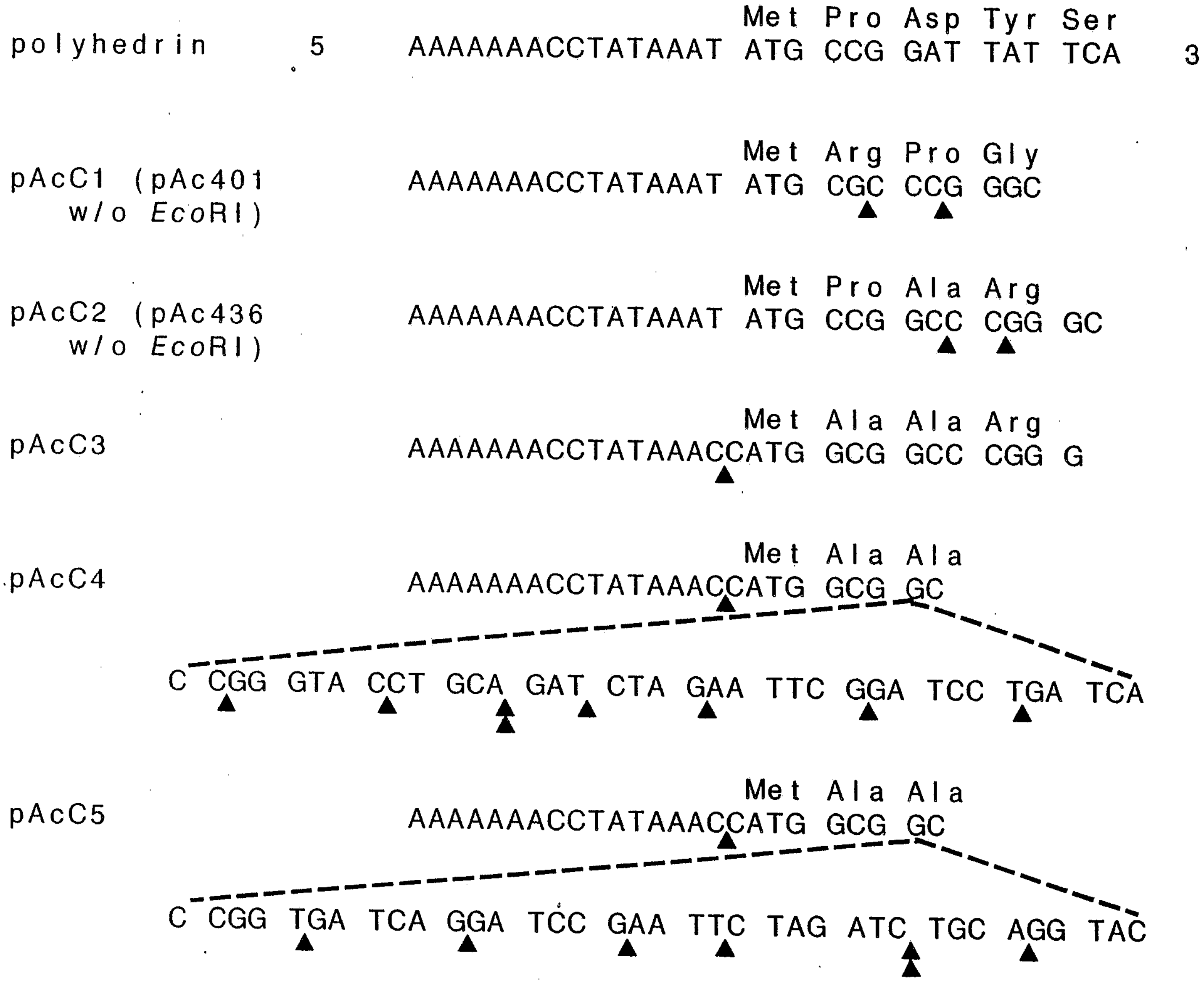

polyhedrin
5
Met Pro Asp Tyr Ser
AAAAAAACCTATAAAT ATG CCG GAT TAT TCA
3
pAcC1 (pAc401 w/o *Eco*RI)
Met Arg Pro Gly
AAAAAAACCTATAAAT ATG CGC CCG GGC
pAcC2 (pAc436 w/o *Eco*RI)
Met Pro Ala Arg
AAAAAAACCTATAAAT ATG CCG GCC CGG GC
pAcC3
Met Ala Ala Arg
AAAAAAACCTATAAACCATG GCG GCC CGG G
pAcC4
Met Ala Ala
AAAAAAACCTATAAACCATG GCG GC
C CGG GTA CCT GCA GAT CTA GAA TTC GGA TCC TGA TCA
pAcC5
Met Ala Ala
AAAAAAACCTATAAACCATG GCG GC
C CGG TGA TCA GGA TCC GAA TTC TAG ATC TGC AGG TAC

FIG. 4 BACULOVIRUS TRANSFER VECTORS

FIG. 5

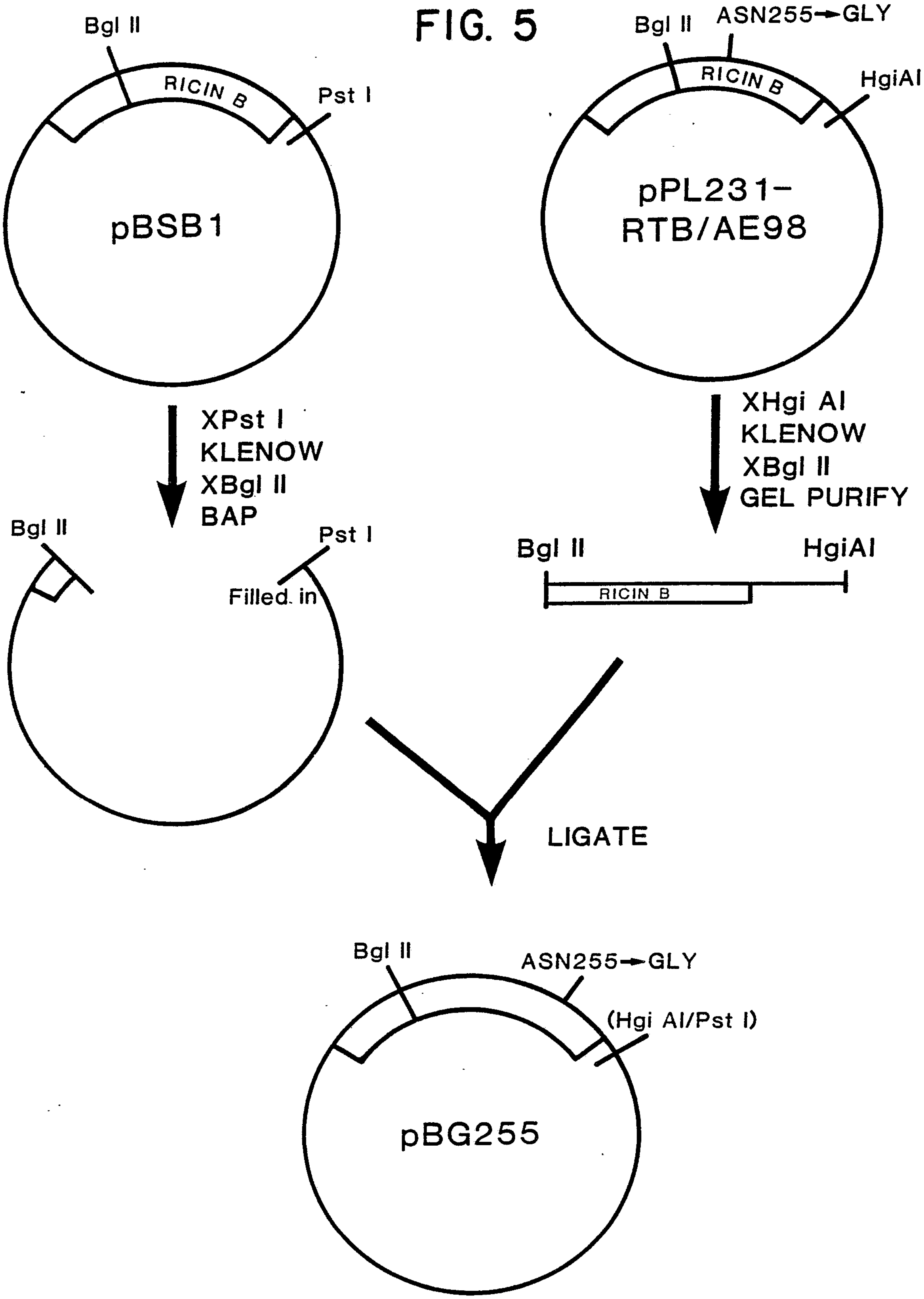

Bgl II
RICIN B
Pst I
pBSB1
Bgl II
ASN255→GLY
RICIN B
HgiAI
pPL231-
RTB/AE98
XPst I
KLENOW
XBgl II
BAP
XHgi AI
KLENOW
XBgl II
GEL PURIFY
Bgl II
Pst I
Filled in
Bgl II
HgiAI
RICIN B
LIGATE
Bgl II
ASN255→GLY
(Hgi AI/Pst I)
pBG255

FIG. 6

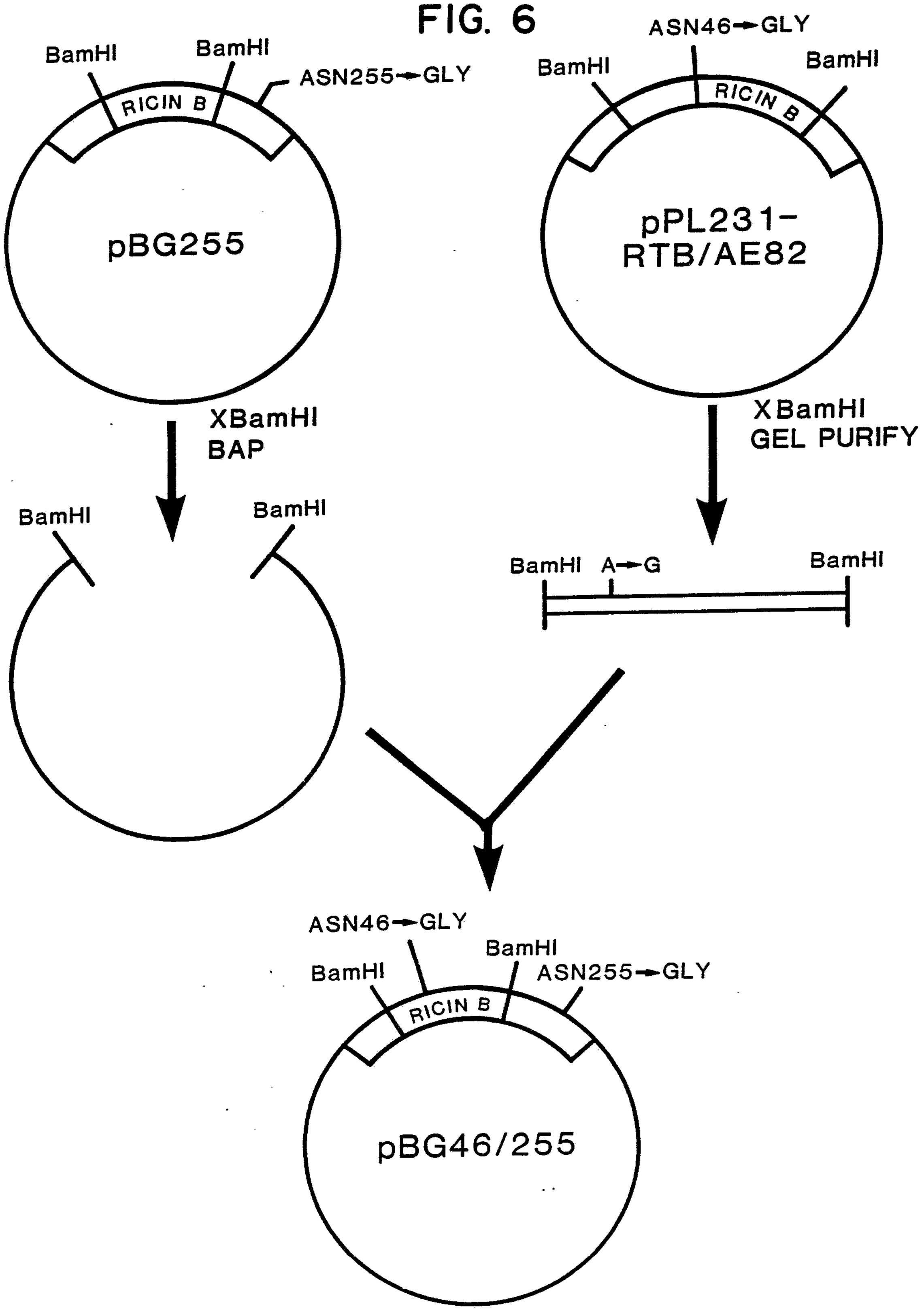

BamHI
BamHI
ASN255→GLY
RICIN B
pBG255
ASN46→GLY
BamHI
BamHI
RICIN B
pPL231-
RTB/AE82
XBamHI
BAP
XBamHI
GEL PURIFY
BamHI
BamHI
BamHI
A→G
BamHI
ASN46→GLY
BamHI
BamHI
ASN255→GLY
RICIN B
pBG46/255

FIG. 7A

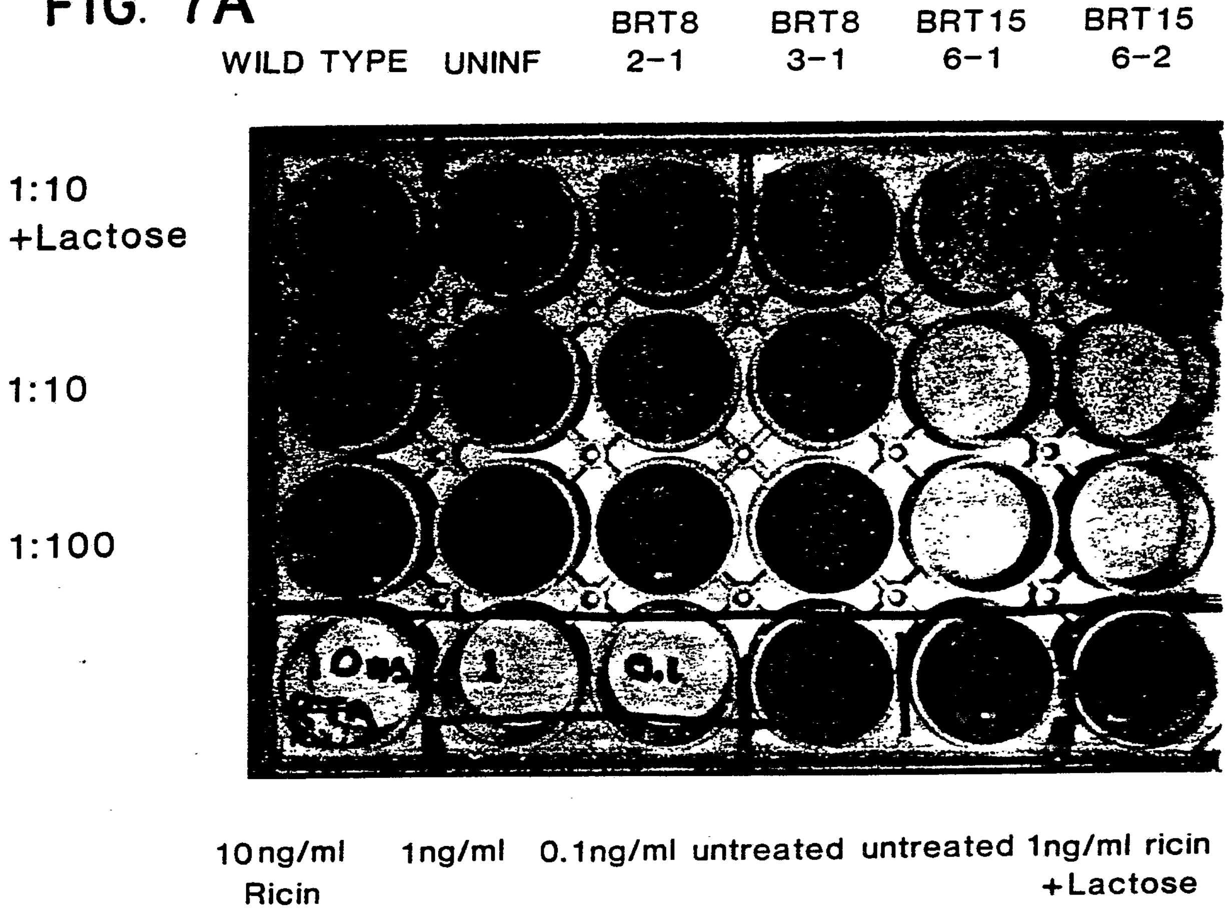

WILD TYPE
UNINF
BRT8 2-1
BRT8 3-1
BRT15 6-1
BRT15 6-2
1:10 +Lactose
1:10
1:100
10ng
1
0.1
10ng/ml Ricin
1ng/ml
0.1ng/ml
untreated
untreated
1ng/ml ricin +Lactose

FIG. 7B

same as above

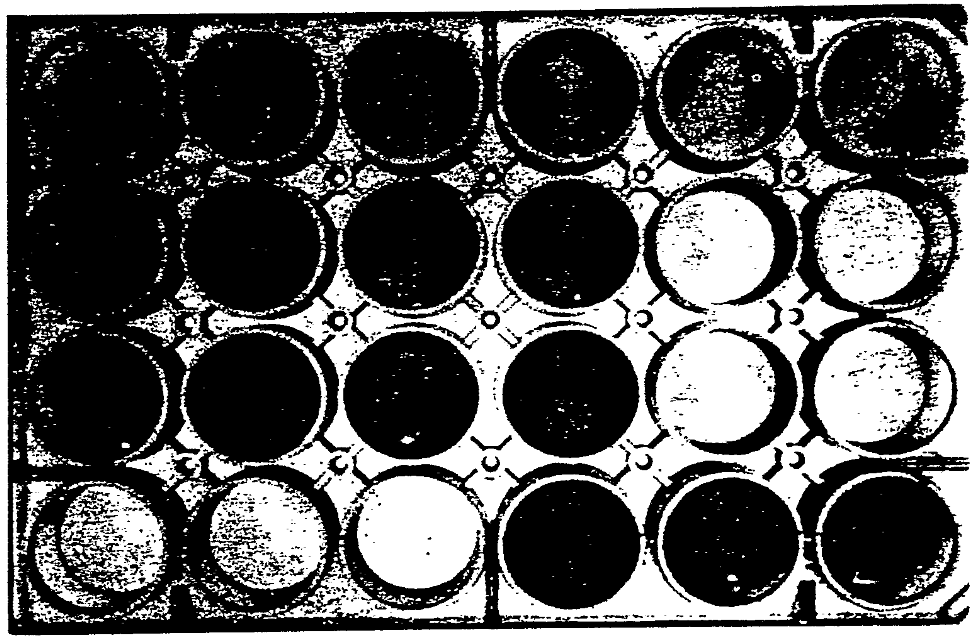
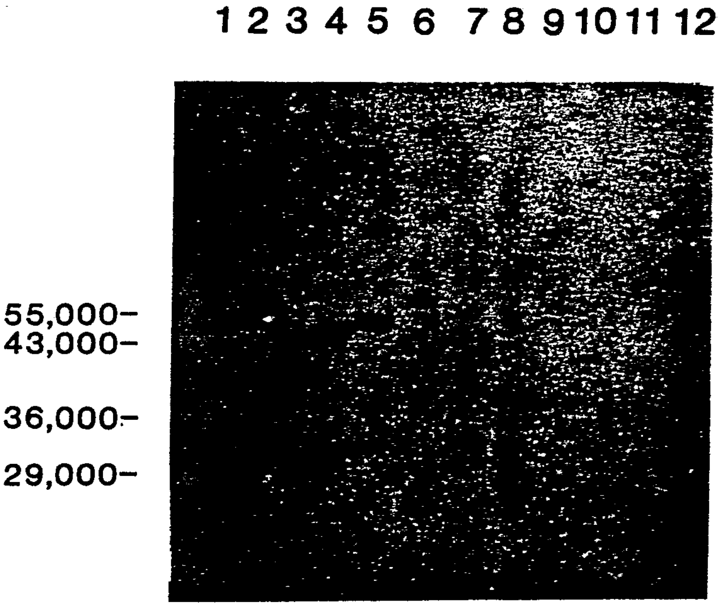


FIG. 8


1 2 3 4 5 6 7 8 9 10 11 12
55,000–
43,000–
36,000–
29,000–

Probed with anti-RA antibody

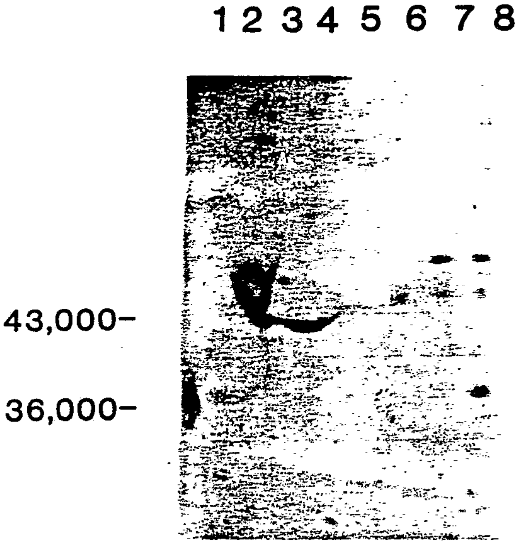
Lane

1. Ricin, 0.5 µg
2. Mol. wt. markers
3. Antigen test
4. Antigen test
5. Wild type
6. BRT8 2-1
7. BRT15 6-1
8. BRT15 6-2
9. BRT8 2-1
10. BRT15 6-1
11. BRT15 6-2
12. Mol. wt. markers

Lanes 5–8: 10µl Immnoprecipitated

Lanes 9–11: 100µl Immnoprecipitated

FIG. 9



Probed with
anti-RB antibody

Lane

1. Ricin 0.5 µg
2. Mol. wt. markers
3. Antigen test
4. Antigen test
5. Wild-type
6. BRT 8-2-1
7. BRT 15-6-1
8. BRT 15-6-2

(lanes 5–8) } 100µl Immnoprecipitated

FIG. 10

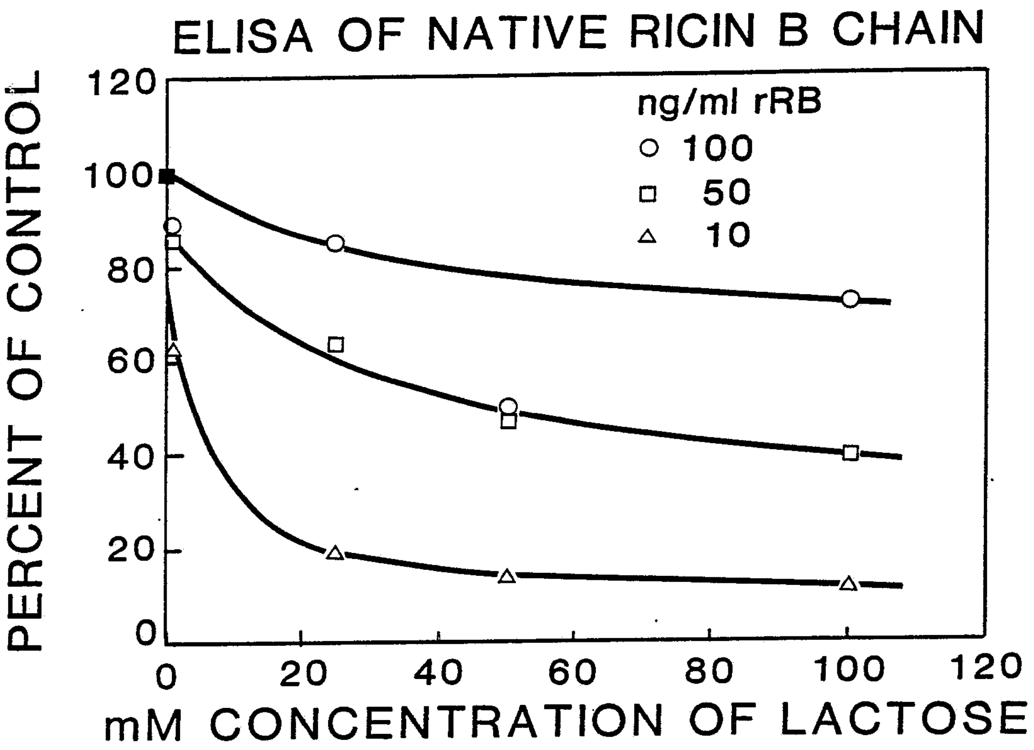
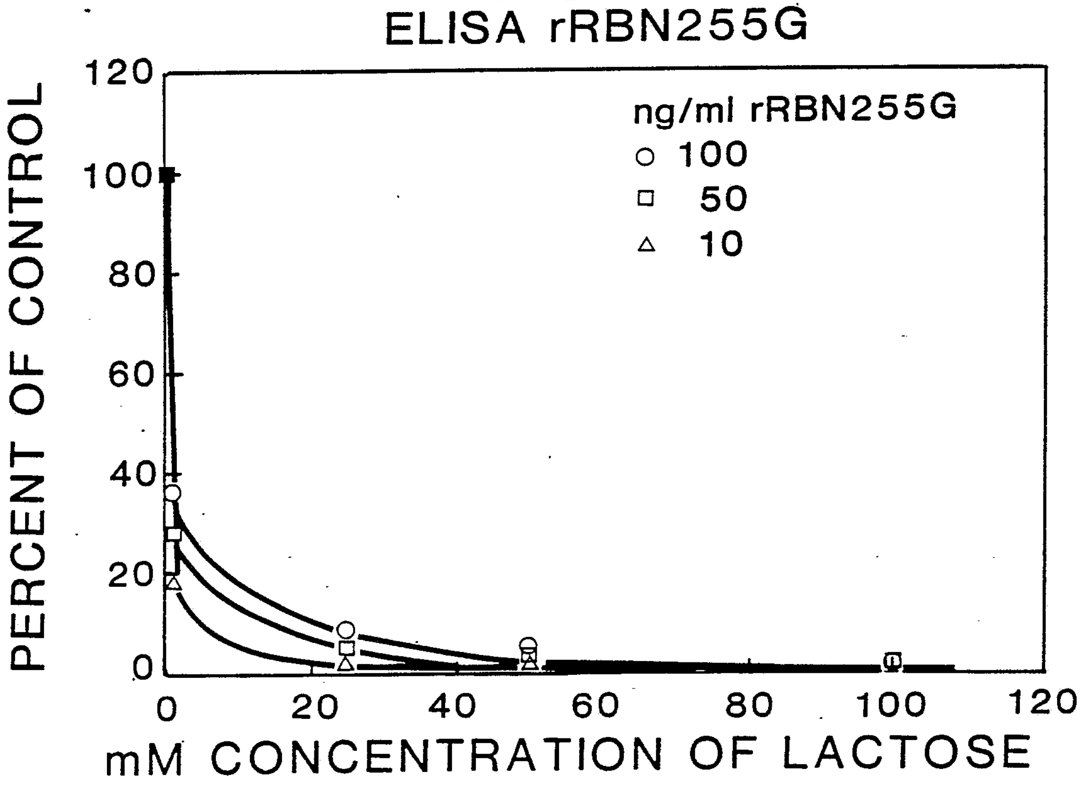

ELISA OF NATIVE RICIN B CHAIN
PERCENT OF CONTROL
0
20
40
60
80
100
120
mM CONCENTRATION OF LACTOSE
ng/ml rRB
○ 100
□ 50
△ 10

FIG. 11


ELISA rRBN255G
PERCENT OF CONTROL
0
20
40
60
80
100
120
mM CONCENTRATION OF LACTOSE
ng/ml rRBN255G
○ 100
□ 50
△ 10

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/02442

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: C 12 N 15/00; C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^4	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0145111 (THE UNIVERSITY OF WARWICH) 19 June 1985 see claims cited in the application --	1,3,33
Y	EP, A, 0127839 (THE TEXAS A&M UNIVERSITY SYSTEM) 12 December 1984 see claims --------	1,3,33

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 31th October 1988	Date of Mailing of this International Search Report 25. 11. 88
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer P.C.G. VAN DER PUTTEN

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 8802442

SA 23639

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 15/11/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0145111	19-06-85	JP-A- 60102188	06-06-85
EP-A- 0127839	12-12-84	AU-A- 2871784	29-11-84
		JP-A- 60037988	27-02-85
		CA-A- 1222213	26-05-87
		US-A- 4745051	17-05-88

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82